Mechanistic insight into the clonal expansion advantage of quiescent preneoplastic breast cells

A thesis submitted to the faculty of the University of Minnesota by

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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May 2018

Table of Contents

Declaration ..... **vi**

Acknowledgements ..... **vii**

Abstract ..... **viii**

Table of Figures ..... **x**

Chapter 1: Introduction **1**

1.1 Breast Cancer Prevalence **1**

1.2 Preneoplastic Lesions **3**

1.3 Thesis Aims **6**

Chapter 2: Materials and Methods **7**

2.1 Buffers and Solutions **7**

2.1.1 Protein Lysis Buffer 7

2.1.2 Western Blot Buffers 7

2.1.3 RNA Isolation Buffers 8

2.1.4 Organoid Immunofluorescent Reagents 8

2.2 Cell Culture **9**

2.2.2 MCF10A/MCF12A Growth Media 9

2.2.3 MCF10A Resuspension Media 9

2.2.4 3D Cell Culture Media Base 10

2.2.5 3D Cell Culture Media + EGF 10

2.2.6 NIH-3T3 Growth Media 10

2.2.7 T47D Growth Media 10

2.3 3D Cell Culture Studies 11

2.4 Cells 12

2.4.1 Cell Lines 12

2.4.2 Viral Vectors 12

2.5 Western Blot 13

2.5.1 Protein lysis, quantification, and sample preparation 13

2.5.2 Running, transfer, detection 13

2.6 Immunohistochemistry staining 14

2.7 Molecular Biology Techniques 14

2.7.1 Virus production 14

2.7.2 Isolation and Quantification of RNA 15

2.7.3 Generation of cDNA and Gene Detection by quantitative PCR 15

2.7.5 Soft Agar Assay 16

2.9 Antibodies 16

2.10 Statistical Analysis 17

Chapter 3: Preneoplastic Quiescent Model System 19

3.1 Introduction 19

3.2 Model System 22

3.2.1 Cell Lines 22

3.2.2 Three-Dimensional Cell Culture 23

3.2.3 Modeling Preneoplastic Cells 23

3.2.4 Entire Model System of Preneoplastic, Quiescent Cells 24

3.2.5 Preneoplastic Cells Remain Quiescent 25

3.2.6 Other Models for Preneoplastic Cells 26

3.3 Discussion 27

3.4 Figures 29

Chapter 4: Quiescence Maintenance via Cell-Intrinsic Feedback Signaling 34

4.1 Introduction 34

4.2 Results 34

4.2.1 EGF is not Sufficient to Promote Outgrowth 56

4.2.2 Cell-Cycle Machinery Expression Differs from Normal Cells 37

4.2.3 Genetic Expression Differs Between Proliferating and Quiescent Cells 38

4.2.4 BGN Over-Expression Decreases Cell Proliferation 40

4.3 Discussion 41

4.4 Figures 44

Chapter 5: Microenvironment Interactions Promotes Clonal Expansion Advantage 52

5.1 Introduction 52

5.2 Results 56

5.2.1 CAFs Promote Cell-Cycle Re-Entry 56

5.2.2 CXCL12 Signaling Drives CAF-Promoted Cell-Cycle Re-Entry 57

5.2.3 CXCL12 Promotes Cell-Cycle Re-Entry Through PKA and MEK via CXCR4 59

5.2.4 Increased CXCR4 Expression Favors Breast Tumor Initiation and Progression 61

5.3 Discussion 62

5.4 Figures 65

Chapter 6: Conclusion 80

6.1 Introduction 80

6.2 Modeling Preneoplastic Quiescence 81

6.3 Maintenance of Quiescence 83

6.4 Clonal Expansion Advantage 85

6.5 Overall Implications 88

References: 90

Declaration

I, Zahra Masoud, hereby certify that this thesis, which is approximately 16,000 words in length, has been written by me, that it is the record of work carried out by me, and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in August 2012 and as a candidate for the Doctor of Philosophy degree in August 2013. This thesis is a record for the work that I carried out at the University of Minnesota between 2013 and 2018.

Acknowledgements

This project would not have been possible without the support of many people. First and foremost, I would like to thank my adviser, Dr. Cheuk Leung. He was truly the best mentor and role model I could have asked for. Without his understanding, patience, and kindness this all would not have been possible. Next, I would like to thank my committee members: Dr. Sundaram Ramakrishnan, Dr. Hiroshi Hiasa, Dr. Subree Subramanian, and Dr. Kaylee Schwertfeger. They have been so helpful throughout my tenure with advice and recommendations. Third, I thank the University of Minnesota Department of Pharmacology. Finally, I would like to thank my family and friends for being so supportive of me while I pursued my PhD. It truly means a lot.

Abstract

Breast cancer, along with most cancers, is preceded by a precancerous state. The majority of cancer research is focused on understanding and targeting cells once they have reached the neoplastic stage. Advancements in early detection have identified the presence of preneoplastic lesions in the breast reporting that cells in these lesions remain phenotypically normal but have pro-growth genetic alterations. These emerging preneoplastic cells carry oncogenic drivers are significantly predisposed to developing tumors, but can stay in a latent state resembling normal quiescent cells in premalignant tissues. Expansion of such preneoplastic populations underlies a key cellular process driving premalignant development and is a hallmark in precancerous lesions and premalignant tissue fields surrounding tumors of epithelial origins such as the breast. Despite the indolent nature, preneoplastic clonal expansion is crucial to propagating and facilitating the accumulation of oncogenic alterations that drive clonal evolution of tumorigenesis and ultimately leads to neoplastic progression. The cellular controls of preneoplastic clonal expansion during premalignant development remain largely unknown. A major barrier to investigating preneoplastic clonal expansion is the lack of experimental models to recapitulate quiescent preneoplastic cells in premalignant tissues. Throughout my PhD research, I have devised a three-dimensional organoid model of quiescent preneoplastic breast cells. Using this model system, I have found that breast cells with aberrant AKT

activation have distinct molecular controls that contribute to an altered homeostatic state of quiescence. This altered state underlies the maintenance of growth-arrest under normal conditions and the proliferative advantage in suppressive tissue environments.

Table of Figures

Figure 1: Schematic of inducible cassettes and time-line of organoid culture models. 29

Figure 2: Representative pictures of Day 20 organoid culture. 30

Figure 3: Representative images of activated AKT in organoid. 31

Figure 4: Organoid proliferation images and quantification of MCF10A myrAKT1 cells. 32

Figure 5: Organoid proliferation of MCF10A AKT-E17K and MCF12A myrAKT1 cells. 33

Figure 6: MCF10A myrAKT1 cells up-regulate p57Kip2 protein expression……44

Figure 7: MCF10A myrAKT1 cells up-regulate p57Kip2 mRNA expression…….45

Figure 8: p57Kip2 knock-down promotes cell cycle re-entry of MCF10A myrAKT1 cells……..……46

Figure 9: MCF10A myrAKT1 Quiescent vs. Proliferating mRNA Heatmap……..47

Figure 10: MCF10A myrAKT1 over-expresses BGN RNA……..48

Figure 11: BGN knock-down increases proliferation in MCF10A myrAKT1 cells49

Figure 12: BGN knock-down does not promote cell transformation……..……50

Figure 13: BGN over-expression inhibits growth of breast tumor cells……..51

Figure 14: Cells remain growth-arrest despite different EGF concentrations…..65

Figure 15: CAF conditioned media promotes outgrowth of MCF10A myrAKT1 cells 66

Figure 16: CAF-secreted CXCL12 mediates outgrowth of MCF10A myrAKT1 cells 67

Figure 17: Recombinant CXCL12 promotes proliferation of MCF10A myrAKT1 cells 68

Figure 18: Recombinant CXCL12 selectively promotes outgrowth in AKT mutated cells 69

Figure 19: MCF10A myrAKT1 cells up-regulate CXCR4 protein and mRNA. 70

Figure 20: AMD3100 prevents CXCL12-mediated outgrowth. 71

Figure 21: CXCR4 over-expression in control cells promotes outgrowth..... 72

Figure 22: AMD3100 blocks CAF-mediated outgrowth of MCF10A myrAKT1 cells. 73

Figure 23: AMD3100 blocks CXCL12-mediated outgrowth in AKT-mutated cells. 74

Figure 24: CXCL12 promotes outgrowth through PKA and MEK. 75

Figure 25: CXCL12 promotes outgrowth of single-infected myrAKT1 cells 76

Figure 26: CXCL12-mediated outgrowth does not transform MCF10A cells. 77

Figure 27: Increased CXCR4 expression correlates with premalignant breast lesions. 78

Figure 28: Model of selective clonal expansion of myrAKT1 cells..... 79

Chapter 1: Introduction

1.1 Breast Cancer Prevalence

About 1 in 8 women in the United States will develop invasive breast cancer over the course of her lifetime. It is the most commonly diagnosed cancer among women and accounts for approximated 26% of all incident cancers among women[49,52]. Each year 400,000 women die of breast cancer, making it the second-leading cause of cancer deaths among American women after lung cancer. The lifetime risk of dying of breast cancer is approximately 3.4%[52]. The international incidence of breast cancer in women varies drastically. While the highest incidence occurs in the United States and Northern Europe, the lowest incidence occurs in Asia[52]. Unfortunately, incidence rates have been rising in Asian countries in recent years as these regions make the transition towards a Western-style economy and pattern of reproductive behavior. Age and family history can affect the risk of breast cancer development[52,56]. Age sharply increases breast cancer incidence rates becoming substantial before 50 years of age[52]. Although breast cancer cannot be prevented currently, early detection can increase treatment options and chances of survival. The size and spread of breast tumors are two of the most important factors in predicting the prognosis or outlook of a women with the disease. Women whose breast cancer is detected at an early stage have a 93%+ survival rate in the first five years[52].

Cancer prevention would have a profound impact on cancer-associated mortality and morbidity but it requires in-depth knowledge of physiological processes underlying tumor initiation[57]. Tumorigenesis of breast cancer, and many other cancers, is considered a multi-step and linear progression through sequential stages of preneoplastic lesions, neoplastic lesions, and ultimately invasive cancer[56,57]. A number of changes occur in cells as they progress towards a greater degree of malignancy. During this process, genomic instability occurs, followed by alterations in oncogenes, onco-suppressor genes, and DNA repair genes, along with surface alterations and alterations in intercellular interactions which leads to the consequent changes in the signal transduction network[49,63]. The progressive accumulation of genetic changes generates autonomously growing neoplastic lesions[49]. Cells in these lesions are both genetically and phenotypically altered[49,56]. The relationship between these changes and the progression towards neoplasia is not understood.

One of the major challenges of cancer research has been the discovery of tools for efficient prevention of malignancy[49,56]. The identification of very early biochemical, molecular, and morphologic changes that predisposes normal cells to malignant transformation plays a pivotal role. In principle, tumor prevention would imply the protection against tumor initiation or an efficacious preventative strategy to block the evolution of initiated cells to malignancy. Early identification of pre-neoplastic lesions is a prerequisite for efficacious prevention.

1.2 Preneoplastic Lesions

Pre-neoplastic breast lesions represent a broad spectrum of lesions with a variable risk of progression to carcinoma[59,63]. Various types of preneoplastic lesions have been identified in breast tissue. Common pre-neoplastic breast lesions seen in benign breast lesions are lobular neoplasia in situ (LIN), papilloma, atypical hyperplasia (AH), and flat epithelial atypia (FEA)[59]. In benign breast lesions which show associated findings of preneoplastic lesions, genetic alterations begin to occur quite quickly leading to unbalanced proliferative and apoptotic signals that may initiative neoplastic proliferation[59]. Regulation of cell proliferation is a vital physiologic phenomenon that helps to prevent malignant transformation within a cell population[56,59]. The evolution of clonal expansion of preneoplastic cells in preneoplastic lesions pushes these lesions to progress into more cancerous lesions[1]. Although the level of proliferation seen in more progressed lesions is very high and disorganized, proliferative levels in preneoplastic cells remain fairly low. For example, previous studies looked at the proliferation rates of preneoplastic atypical hyperplasia lesions in the breast, the median value for percent positive cells for Ki67 was 1.0% with the 75th percentile being 2.3%[59]. This highlights that preneoplastic lesions contain preneoplastic quiescent cells that have an advantage to clonally expand but are phenotypically different than neoplastic cells.

There is no way to determine the presence of a preneoplastic lesion by reviewing a mammogram or other breast imaging study[59,64]. Improvements in clinical radiology and the routine ruse of large core needle biopsies have led to an increased detection rate of early precursor lesions in the breast[64]. This has provoked questions regarding the role of these lesions in the evolution of breast cancer, specifically whether the presence of preneoplastic lesions increases the risk of breast cancer development in the future. Results exploring this risk concluded that the presence of a preneoplastic lesion in the breast like AH increases the likelihood that breast cancer will develop[49,56]. Specifically, data has shown that 25 years after an AH biopsy, breast cancer (either in situ or invasive) developed in 30% of the women[71,72]. This risk is also increased more if the women have a family history of breast cancer[72].

These advancements of early detection have sparked wanting to know more about premalignant stages. Many studies over the past decades have focused on delineating the genome of preneoplastic lesions. These comprehensive gene-expression studies in breast-cancer progression have shown that similar and progressive transcriptional and epigenetic alterations found in preneoplastic lesions of the breast, are also found in carcinoma in situ and invasive carcinoma of the breast, which provides additional evidence for the precursor role of preneoplastic lesions[49].

Unfortunately, delineating the genome is not sufficient. For example, preneoplastic breast lesions have been reported to exhibit decreased p27[74]

levels and PIK3CA mutations[73]. In addition, it is established that the microenvironment and stroma induce cells to progress to malignancy. The regulation of proliferation versus quiescence is a key determinant of the fate of tumor growth. This critical decision is tightly coupled with interactions between preneoplastic cells, host cells, and immune mechanisms. Specific immune effectors and secreted factors (including cytokines and chemokines) have been implicated in the initiation of tumorigenesis and tumor growth. Similarly, inflammation can be associated with increased tumorigenesis; chronic inflammation has been associated with a poorer breast cancer prognosis[56,71]. Consequently, the presence or absence of immune effectors is associated with a favorable or non-favorable prognosis depending on tissue type, thereby indicating the complexity of the interaction between the host immune system and the evolving tumor[56,71,72]. Additionally, fibroblasts, a major cell type of the microenvironment, have thought to favor tumor progression via secreted factors[75]. Despite the understanding of characteristics associated with breast fibroblasts, further investigation into their role and involvement of the breast cancer microenvironment will yield important and needed insights to the convoluted connections between cancer cells and the stroma. Therefore, understanding how this genome manifests on a cellular and molecular level is needed to gain a better in-depth understanding of preneoplastic cells and lesions. Identifying the underlying mechanisms of how preneoplastic cells exploit the stroma and take advantage of the microenvironment in order to expand will

deepen the understanding of early stages of breast cancer initiation allowing for eventual intervention and prevention.

1.3 Thesis Aims

During my Ph.D. tenure, I have furthered our understanding of the mechanistic insights of the clonal expansion advantage that quiescent, preneoplastic cells have. In Chapter 3, I introduce a model system that is able to recapitulate the genetic and proliferative features of quiescent breast preneoplastic cells and, importantly, provide a platform to interrogate the cellular control of quiescent preneopalstic cells at the mechanistic level. In Chapter 4, I show how regardless of having an oncogenic, pro-growth alteration, preneoplastic cells are able to maintain quiescence is normal conditions through cell-intrinsic feedback signaling mechanisms through altered genetic expression and modulating the microenvironment. In Chapter 5, I show that although the oncogenic alterations do not directly promote proliferation, cells are sensitive to non-native growth factors priming the cells to proliferate under specific environments that involve genetic and microenvironment interactions. Finally, in Chapter 6 I discuss that my research suggests that altered homeostatic controls of quiescence may also underlie the maintenance and selective clonal expansion of indolent preneoplastic cells during premalignant development and what the overall impact and implications of these findings represent.

Chapter 2: Materials and Methods

2.1 Buffers and Solutions

2.1.1 Protein Lysis Buffer

RIPA Buffer (ThermoFisher Scientific)

PBS (ThermoFisher Scientific)

Halt Protease and phosphatase inhibitor (ThermoFisher Scientific)

2.1.2 Western Blot Buffers

Western Blot 4X Reducing Sample Buffer

Laemmli's SDS-Sample Buffer (4X, Reducing) (Boston BioProducts)

Western Blot Running Buffer

Tris-Glycine-SDS Running Buffer (10X) (Boston BioProducts)

Western Blot Transfer Buffer

Transfer Buffer (10X) (Boston BioProducts)

10% Methanol (ThermoFisher Scientific)

Western Blot Wash Buffer

Tris-Buffered Saline (20X) (Boston BioProducts)

0.1% Tween-20 (ThermoFisher Scientific)

Western Blot Stripping Buffer

Membrane Stripping Buffer (4X) (Boston BioProducts)

2.1.3 RNA Isolation Buffers

RNeasy Mini Kit (Qiagen)

TRIzol Reagent (Ambion Life Technologies)

2.1.4 Organoid Immunofluorescent Reagents

Fixation Buffer: 4% PFA

Paraformaldehyde, 16% (Ted Pella, Inc.)

PBS

1X PBS/Glycine Buffer

3.75 g Glycine

500 mL PBS

10X IF Wash Buffer

2.5 g NaN_3

5 g BSA

10 mL TritonX-100 (ThermoFisher Scientific)

2.5 mL Tween-20 (ThermoFisher Scientific)

500 mL 10X PBS

1X PBS/0.5% Triton-X Buffer

2.5 mL Triton X-100 (ThermoFisher Scientific)

500 mL 1X PBS

2.2 Cell Culture

2.2.2 MCF10A/MCF12A Growth Media

500 mL DMEM/F12 Medium (ThermoFisher Scientific)

25 mL Horse Serum (ThermoFisher Scientific)

100 µL EGF (100 µg/ml) (Peprotech)

250 µL Hydrocortisone (1 mg/mL) (Sigma-Aldrich)

50 µL Cholera Toxin (1 mg/mL) (Sigma-Aldrich)

500 µL Insulin (10 mg/mL) (Sigma-Aldrich)

2.5 mL Penicillin/Streptomycin (200X) (ThermoFisher Scientific)

2.2.3 MCF10A Resuspension Media

400 mL DMEM/F12 Medium (ThermoFisher Scientific)

100 mL Horse Serum (ThermoFisher Scientific)

2.5 mL Penicillin/Streptomycin (200X) (ThermoFisher Scientific)

2.2.4 3D Cell Culture Media Base

500 mL DMEM/F12 Medium (ThermoFisher Scientific)

10 mL Horse Serum (ThermoFisher Scientific)

250 µL Hydrocortisone (1 mg/mL) (Sigma-Aldrich)

50 µL Cholera Toxin (1 mg/mL) (Sigma-Aldrich)

500 µL Insulin (10 mg/mL) (Sigma-Aldrich)

2.5 mL Penicillin/Streptomycin (200X) (ThermoFisher Scientific)

2.2.5 3D Cell Culture Media + EGF

50 mL 3D Cell Culture Media Base

2.5 µL EGF (100 µg/ml) (Peprotech)

2.2.6 NIH-3T3 Growth Media

500 mL Dulbecco's Modified Eagle's Media (DMEM) (ThermoFisher Scientific)

2.2.7 T47D Growth Media

400 mL RPMI (ThermoFisher Scientific)

10% FBS (ThermoFisher Scientific)

0.01mg/ml Insulin (Sigma-Aldrich)

2.3 3D Cell Culture Studies

Three-dimensional cultures of MCF-10A and MCF12-A cells were set up in 8-well chamber slides (BD Biosciences), or coverglass bottom 8-chamber slides (MatTekII) as previously described with 4500-5000 cells in Assay Media + 2% Matrigel™. Media was replaced every 4 days. To induce oncogene and reporter expression in growth-arrested acini, cultures were treated on Day 16 with fresh media containing 1 ug/mL doxycycline. Inhibitor and chemokine treatments were performed on Day 18 by replacing with fresh media containing 1 ug/mL doxycycline, and the corresponding concentration for inhibitors, chemokines, or vehicle controls. The cultures were analyzed on Day 20.

Single-cell transduction in growth-arrested mammary organoids were conducted as described. On Day 16, 3D cultures were infected with corresponding lentiviruses diluted in Assay Media without EGF for 6-8 hours at 37°C. Virus dosages were adjusted to infect less than 1 cell per 10 acini to achieve sporadic single-cell infection. The viruses were removed, and the chamber wells were rinsed with 500µl PBS and replaced with normal 3D Assay Media without Matrigel. Doxycycline was added at 1µg/mL on the following day along with drug treatment or vehicle control as indicated. Complete media was changed every 4 days. Acinar structures were analyzed eight days after doxycycline induction.

2.4 Cells

2.4.1 Cell Lines

MCF10 cells were a kind gift from Joan Brugge (Harvard Medical School). MCF12A cells were purchased from ATCC. NIH-3T3 and NIH-3T3 CAV-KO cells were obtained from Dr. Zachary Schafer (University of Notre Dame).

2.4.2 Viral Vectors

The lentivector pLT-IGSP (IRES-GFP-sv40-puro) was generated by subcloning a SV40-puro cassette from the pBABE-puro plasmid (Addgene Plasmid #1764) and inserted downstream of IRES-GFP in the pLT-iG lentivector. pLT-iG lentivector containing the tetracycline response element (TRE) from pTre-Tight (Clontech), multiple cloning sites, and a downstream IRES-GFP cassette from pIRES2-GFP (Clontech) were described previously [1]. pLT-AKT-E17K-iGSP was generated by PCR using the hAKT1 coding sequence as template and primers carrying the E17K mutation (5'-taggatccATGAGCGACGTGGCTATTGTGAAGGAGGGTTGGCTGCACAAACGAGGGAAG-3' and 5'-ttACCGGTCTATCAGGCCGTGCCGCTGGCCGAGTAGGAGAACTG-3', and subcloning into the pLT-iGSP vector. Products are confirmed by sequencing. pLT-myrAKT1-iGSP was generated by subcloning the myrAKT1 fragment from pLNCX myrAKT1 (Addgene Plasmid #17245) in the pLT-iGSP vector. pLKO shRNA knockdown constructs were obtained from GE Dharmacon. p57

knockdown clone #39678 (Clone#1) and #10486 (Clone#2) were used. pLT-CXCR4-iGSP is generated by subcloning from pcDNA3.1-DYK-CXCR4 purchased from GenScript. All coding sequences were validated by sequencing.

2.5 Western Blot

2.5.1 Protein lysis, quantification, and sample preparation

Plates for protein harvest were put on ice, washed twice with cold 1xPBS and PBS was removed. Cells were lysed on ice with cold RIPA/Halt and incubated for 15 minutes. Plates were then scraped and lysate was collected into microcentrifuge tube and pulled through a 27-gauge needle 3-5 times. Lysate was incubated on ice for 15 minutes and then spun at maximum speed (>14,000xg) for 10 minutes at 4^{o}C. Supernatant was collected and snap frozen in liquid N_2. Samples were stored at –80^{o}C.

Protein samples were quantified using the Pierce™ BCA Protein Assay Kit (#23227)

Samples were thawed on ice and 15ug of protein, water, and 4x SDS gel loading buffer with DTT (2ME) were combined to total 20ul. Samples were then heated for 5-10 minutes at 95 ^{o}C and set on ice before loaded into the gel.

2.5.2 Running, transfer, detection

Gels were run for 30 minutes at 230V and then immediately proceeded to transfer for 90 minutes at 100mA. Blots were then blocked overnight with

Blocking Buffer. The blot was then incubated with 1:100 dilution of primary antibody for 1 hour, washed, and incubated with 1:5000 HRP-conjugated secondary antibody for 1 hour.

ECL reagent (Luminata Crescendo) was added to membrane for detection.

2.6 Immunohistochemistry staining

Immunofluorescent analyses were performed as previously described. Briefly, cells were fixed at room temperature for 30 minutes with 4% paraformaldehyde. Primary antibodies were used at 1:100-1:200 and incubated overnight in the dark at room temperature. Alexa secondary antibodies (ThermoFisher Scientific) were used at 1:200 and incubated for one hour at room temperature. Nuclei were counterstained with DAPI. Images were acquired using a Nikon A1RMP confocal microscope in the University Imagine Center at the University of Minnesota.

2.7 Molecular Biology Techniques

2.7.1 Virus production

Retroviruses and lentiviruses were produced by co-transfecting the corresponding viral vectors with packaging vectors pCL-Ampho (retroviruses, Imgenex) or psPAX2 and pMD2.G (lentiviruses, Addgene #12260 and #12259) into 293T cells with TurboFECT (ThermoFisher Scientific). Virus-containing

supernatants were collected on day 2 and 3 following transfection and were stored at −80°C.

2.7.2 Isolation and Quantification of RNA

RNA was collected with TRizol and Chloroform and spun at 10,000xg for 18 minutes at 4°C. The aqueous phase was removed and combined with 100% RNA-free Ethanol. The protocol provided with the RNAeasy Kit was then followed.

2.7.3 Generation of cDNA and Gene Detection by quantitative PCR

3D cells were incubated on ice in Cell Recovery Solution (Corning) for 30 minutes and washed. RNA was extracted by TRIzol RNA Isolation Reagents (ThermoFisher Scientific), and purified using RNeasy (Qiagen). First strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System according to the vendor protocol. qPCR was perfomed using SYBR Select Master Mix and StepOnePlus Real Time PCR System (ThermoFisher Scientific). 0.2uM primer concentration was used. The following primer sequences were used:

Transcripts were considered to be undetectable when the Ct value was above 32. qPCR was performed using three individual biological samples in duplicate.

ΔΔCt was calculated by normalizing to RPLR0 and average and standard deviation were determined. Statistically significant differences were determined by Student's t-test.

2.7.5 Soft Agar Assay

10,000 cells in 1.5 mL 0.4% Sea Plaque low melt agarose was layered on top of a 2 mL 0.5% agarose base layer per well of a 6-well plate. Triplicate samples were used. 1 mL of growth media with 1 ug/mL doxycycline and CXCL12 or PBS was overlaid on the solidified cells/agarose mix. Media was changed every 3 days for 30 days.

2.9 Antibodies

Antibody	Company	Catalog Number	Species
p57	Santa Cruz Biotechnology	SC-1040	Rabbit
Ki67	ThermoFisher Scientific	180191Z	Rabbit
CXCR4	R&D Systems/ Bio-Techne	MAB 172-100	Mouse

Beta Actin	Sigma Aldrich	A1978	Mouse
BGN	Sigma Aldrich	HPA003157	Rabbit
Cleaved Caspase 3	Cell Signaling Technology	9661S	Rabbit
pAKT	Cell Signaling Technology	4060S	Rabbit

2.10 Statistical Analysis

All cell counting experiments were collected from 3 individual experiments with 100 total number of isolated GFP+ organoid structures. Percent of total number of Ki67+ cells per nuclei in each organoid were recorded under a Leica DMI 3000 fluorescent microscope. Average and SEM (Standard Error Means) of the percent of Ki67+ cells were calculated. Statistically significant differences were determined by two-tail Student's t-test and were used for pairwise comparison.

For clonal expansion experiments, acini were fixed in 4% PFA for 20 minutes at room temperature and counterstained for nuclei with DAPI. Isolated, infected acinar structures were scored for expansion and for spatial location of

the infected cells/clones with respect to the structures. GFP-labeled cell clusters containing more than one nucleus based on DAPI counterstain were scored as expanded clones. Three individual experiments with 80-120 acini counted each were analyzed per assay. Means and standard deviations were calculated. Statistical significance was determined by two-tail Student's t-test.

Chapter 3: Preneoplastic Quiescent Model System

3.1 Introduction

Cancer models are the primary tool used by cancer researchers in order to test specific hypothesis related to cancer as they allow systemic manipulation of known parameters. Ideal models recapitulate both the cellular and molecular mechanisms driving cancer progression, can be manipulated to precisely answer questions about these mechanisms, and provide reproducible results over a period of time.

With one in eight American women at risk of developing breast cancer within her lifetime, understanding the underlying mechanisms that drive breast cancer progression is crucial. Neoplastic transformed cells exhibit some of the features involved in multi-step carcinogenesis such as morphological changes, lack of contact inhibition, independence of their growth from specific growth factors, oncogene activation, and tumor suppressor gene inactivation. The earliest lesions and genetic transitions usually occur years before a tumor is detected. While early stage ductal carcinoma in situ (DCIS) is not considered life threatening with a 10-year survival rate of about 90%, this drops dramatically to under 10% when the cancer is detected at later stages. Based on previous studies over the past decades, early detection of breast cancer might provide the greatest opportunity for a cure. Unfortunately, little is known about the underlying genetic events that trigger the progression of a normal cell into a cancerous one.

Therefore, it is imperative to understand the earliest events that trigger this progression. The identification of biomarkers and development of selective therapeutics targeting key pathways in preneoplastic cells will represent a “holy grail” in breast cancer treatment and prevention.

The mammary gland is composed of an organized ductal network. Embedded within the stroma, the branching duct system leads from the collecting ducts via the segmental and subsegmental ducts to the terminal duct lobular units (TDLUs). Two cell types compose the epithelium of the duct and lobule system, namely luminal (secretory) cells and myoepithelial cells. Almost all mammary carcinomas develop within the TDLU or the terminal ducts that enter the lobular units.

The regulation of proliferation versus quiescence is a key determinant of the fate of tumor growth. While premalignant and pre-invasive breast lesions are relatively common, only a small percentage progress to high grade invasive breast cancer. Therefore, important biological differences must exist between those that remain stable and those that progress into cancer. Emerging preneoplastic cells that carry oncogenic drivers are significantly predisposed to developing tumors, but can stay in a latent state resembling normal quiescent cells in premalignant tissues.

Currently, little is known about the biology of quiescence and a number of obstacles have hindered the study of early tumor progression. This reflects the

difficulties in identifying and isolating preneoplastic, quiescent cells in humans, as well as the general lack of *in vitro* experimental systems and scarcity of *in vivo* models. In the past, the lack of experimental systems has greatly hampered the mechanistic study of preneoplastic quiescent cells. A major barrier to investigating preneoplastic clonal expansion is the lack of experimental models to recapitulate quiescent preneoplastic cells in pre-malignant tissues. Non-transformed cell lines are amenable to genetic manipulation to mimic the molecular context of preneoplastic cells, but engineered cells with oncogenic alterations tend to proliferate under traditional culture conditions. Tissue environment also plays crucial roles in suppressing preneoplastic cells in native tissues of animal models[2-4], and therefore significantly impedes mechanistic study. Modeling pre-malignancy in vitro is complex and has only been made possible in the last decade through the advent of three-dimensional acini cultures. This model provides a context in which it is feasible to identify genes and dissect mechanisms necessary to produce phenotypic alterations similar to those observed during malignant progression. These can include luminal filling, loss of polarization, and invasive behavior. Although this progress has been made, work is still needed to accurately model pre-malignancy in order to understand the vents that drive a cell towards a cancer fate. Moving forward these models can be used to understand the role of oncogenes that are more common to breast cancer.

Establishing new models to study preneoplastic quiescent cells in a more physiologically relevant environment will lead to a more comprehensive understanding of the underlying mechanism of quiescence. A better understanding of the regulatory mechanisms and conditions that govern the state of preneoplastic quiescence could eventually help identify potential useful markers of these cells and health conditions that could be used to evaluate patients' cancer risks potentially leading to a reduction in cancer mortality.

3.2 Model System

To overcome the limiting hurdles needed to study quiescent preneoplastic cells, I recapitulated the molecular and cellular context of premalignant tissues by combining genetic tools for inducible oncogenic alterations and three-dimensional culture of growth-arrested mammary organoids.

3.2.1 Cell Lines

Since tumor cell lines continue to proliferate under traditional monolayer tissue culture conditions on rigid plastic, I used non-transformed, human mammary epithelial cells (MCF10A and MCF12A) derived from independent individuals[5,6]. These cells carry features particularly suitable for modeling preneoplastic cells. They harbor homozygous deletion at the INK4A/ARF locus[7],

resulting in p16-loss, a common alteration in preneoplastic mammary cells that have bypassed replicative senescence[8,9]. Otherwise, these cells are diploid and genetically stable[10,11]. They have overcome the replication limit, but do not form colonies in soft-agar or tumors in mouse xenografts. Importantly, these mammary cells remain subjected to proliferative constraint exerted by suppressive epithelial environments in organoid cultures[12].

3.2.2 Three-Dimensional Cell Culture

Cells are seeded on Matrigel, reconstituted basement membrane materials that resemble the stiffness and components of the extracellular matrix (ECM) in tissue. When seeded on Matrigel, individual mammary cells proliferate to for spheroids (Day 0-8), undergo luminal clearing (Day 8-12), and develop into growth-arrested organoids that resemble the terminal end bud of the mammary gland (Day 16 onwards) [12]. These orgnaoids remain growth-arrested in complete media containing growth factors and serum[12], recapitulating homeostatic mammary tissues.

3.2.3 Modeling Preneoplastic Cells

Aberrant AKT activation is a major driver of tumor growth[13] that has also been implicated in premalignant development in breast[14-20] and other epithelial

tissues[21]. AKT signaling mediates many cellular processes including cell survival, proliferation, and metabolism; and has important roles in supporting breast tumor growth and progression[22,23]. However, its contribution to premalignant development is less clear. Previous transgenic studies have shown that genetic aberrations that deregulate the PI3K/AKT signaling pathway increase incidence of mammary tumors only after prolonged latency, but significantly promote tumor formation in cooperation with other oncogenic alterations[22,24,25], supporting a role of AKT signaling in premalignant development. Consistent with this notion, aberrant activation of AKT signaling is evident in precancerous breast lesions[14-21,26]. AKT1 is used to model activation of the signaling pathway because it has the broadest specificity for known AKT substrates, is ubiquitously expressed, and has predominant roles in breast tumorigenesis.

3.2.4 Entire Model System of Preneoplastic, Quiescent Cells

To model quiescent, preneoplastic mammary cells with aberrant AKT activation in premalignant tissue, we first engineered cell lines to allow for temporal induction of hyperactive AKT signaling. Parental non-transformed human mammary cells constitutively expressing the reverse tetracycline trans-activator (rtTA) (MCF10A/rtTA or MCF12A/rtTA) were transduced with lentiviral constructs that contain tetracycline (TET)-inducible myrAKT1 (myristolated AKT1, a constitutively active AKT1 variant) and bicistronic IRES (Internal Ribosome-

Entry Site) mediated GFP reporter (pLT-myrAKT1-iGSP), or IRES-GFP control. (Figure 1).

These cells were then cultured on Matrigel in media without doxycycline (Dox, a stable tetracycline analogue) for 16 days to form growth-arrested organoids that recapitulate suppressive premalignant tissue environments. Growth-arrested organoids were then treated with Dox to induce myrAKT1 and GFP reporter expression. These cells have no detectable GFP expression in the absence of Dox and efficiently induce reporter expression upon Dox treatment (Figure 2).

3.2.5 Preneoplastic Cells Remain Quiescent

First, I examined the effects of inducing aberrant AKT activation on growth-arrested organoids. Percent cycling cells per organoid were determined by immunostaining with an antibody against Ki67, a widely used marker in laboratories and clinics for all active cell-cycle phases (non-G_0[quiescent]). I found that the preneoplastic cells in the organoids remain quiescent (0.9±0.2%, Figure. 4) despite significant AKT activation as indicated by increased levels of phosphorylated-AKT on the cell membrane (Figure 3). Maintenance of quiescence was also observed in other preneoplastic mammary cell models based from MCF12A organoids with inducible myrAKT1 expression (1.2±0.2%, Figure 5) and MCF10A organoids with inducible expression of a pathologically

relevant AKT1 mutant in breast cancers[20,29] (AKT1-E17K, 0.6±0.2%) (Figure 5). Moreover, the low proliferation rate is consistent with that reported in normal breast tissues and benign breast lesions (~0.5% to ~3.7%)[16,30], and contrasts the high proliferation rate (37.3±1.8%, Figure 4) of identical cells with AKT signaling induced in actively proliferating spheroids under the same media conditions.

3.2.6 Other Models for Preneoplastic Cells

Interestingly, using similar lentiviral vectors to activate other common oncogenic alterations and co-express a GFP marker, such as c-Myc over-expression or over-expression of CyclinD in single or all cells within the organoids, these preneoplastic cells remain quiescent in culture, similar to the GFP-only expressing control. In contrast, over-expression of ErbB2, a receptor tyrosine kinase that is over-expressed in 25-30% of breast carcinomas, induces outgrowth of quiescent cells.

3.3 Discussion

With breast cancer being one of the most commonly diagnosed cancers among women, cancer prevention would have a profound impact on cancer-associated mortality and morbidity. To potentially achieve this, in-depth knowledge of physiological processes underlying the multi-step and linear progression of tumor initiation is required. In principle, tumor prevention would imply the protection against tumor initiation or an efficacious preventative strategy to block the evolution of initiated cells to malignancy. Therefore, understanding how preneoplastic quiescent cells in preneoplastic lesions are able to maintain quiescence while carrying oncogenic changes and understanding what permissive environments allows them to be advantageous in clonal expansion is needed.

This organotypic system provides a model to study the maintenance of preneoplastic, quiescent cells that contain defined oncogenic alterations. Using cells with aberrant AKT expression as a model for the early genetic changes in preneoplastic cells is representative of all preneoplastic lesions with pro-growth, oncogenic alterations and it allows for insight into the underlying mechanisms of preneoplastic lesions in general. It also permits convenient genetic manipulation and application of non-genetic factors to investigate cellular mechanisms that govern preneoplastic quiescence. Altogether, these organoid models recapitulate the genetic and proliferative features of quiescent breast preneoplastic cells and,

importantly, provide a platform to interrogate the cellular control of quiescent preneoplastic cells at the mechanistic level.

This model system overcomes the major barrier that has impeded investigation of preneoplastic clonal expansion and understanding the underlying mechanisms that regulate and maintain quiescence. Due to its more physiologically relevant environment, a more comprehensive understanding of the regulatory mechanisms and conditions that govern the state of preneoplastic quiescence can be obtained leading to the identification of useful markers of these cells and health conditions. Identifying potential markers and developing prognostic markers could be used to evaluate patients' cancer risks potentially leading to a reduction in cancer mortality and develop stronger tumor prevention strategies.

3.4 Figures

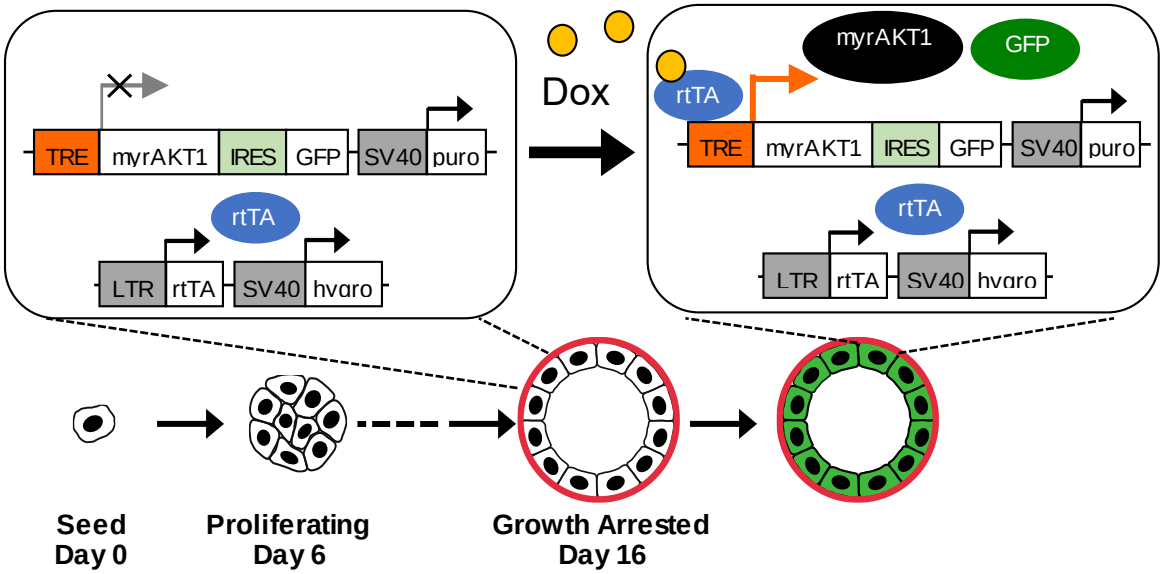

Figure 1: Schematic of inducible cassettes and time-line of organoid culture models.

TRE, tetracycline response element; IRES, internal ribosome entry site; rtTA, reverse tetracycline trans-activator; Dox, doxycycline.

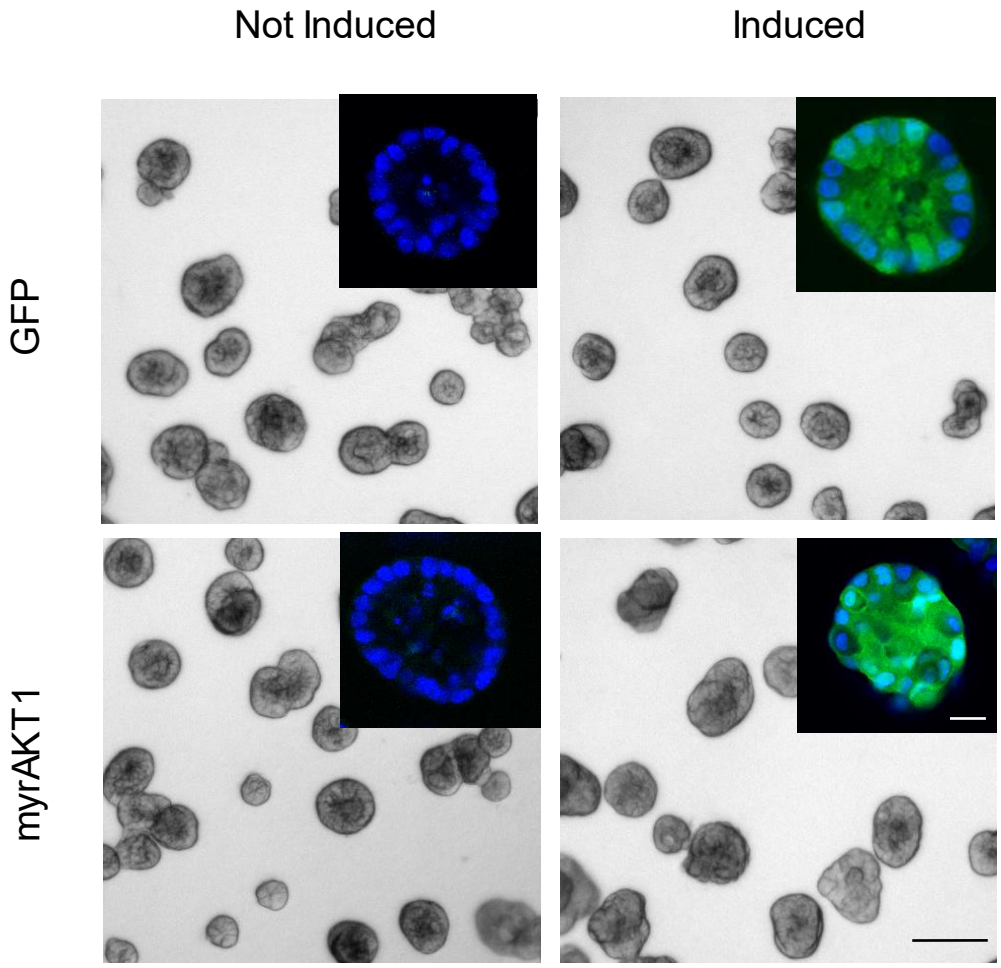

Figure 2: Representative pictures of Day 20 organoid culture.

Scale bar, 50μm. Inlets, individual organoid. Green, GFP reporter. Blue, DAPI nuclear counterstain. Scale bar, 20μm.

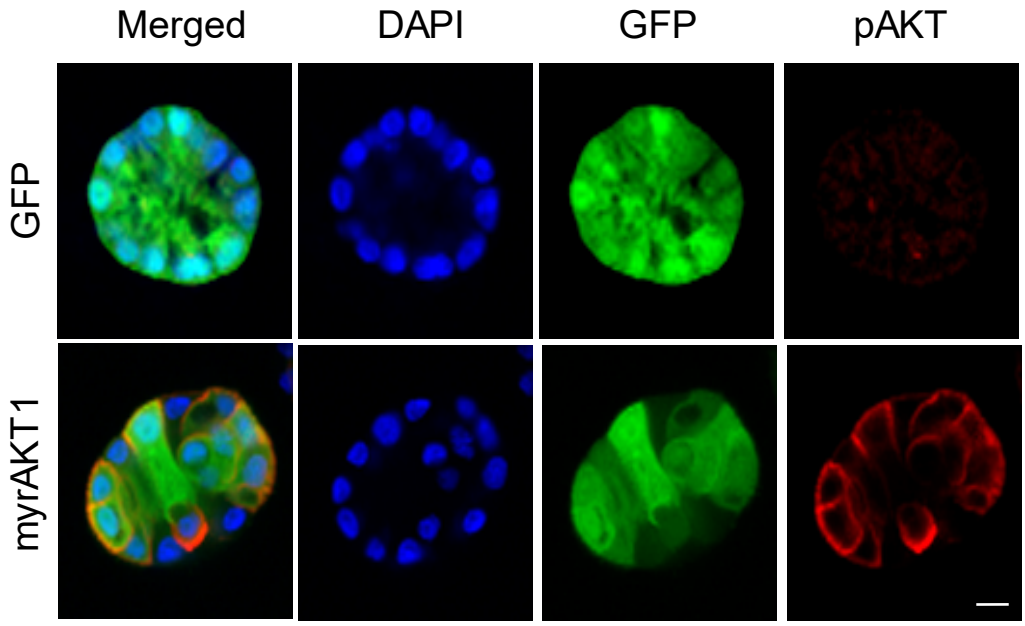

Figure 3: Representative images of activated AKT in organoid.

Representative images of activated AKT (phosphor-AKT, pAKT) in organoid induced with myrAKT1 and GFP reporter or GFP reporter alone.

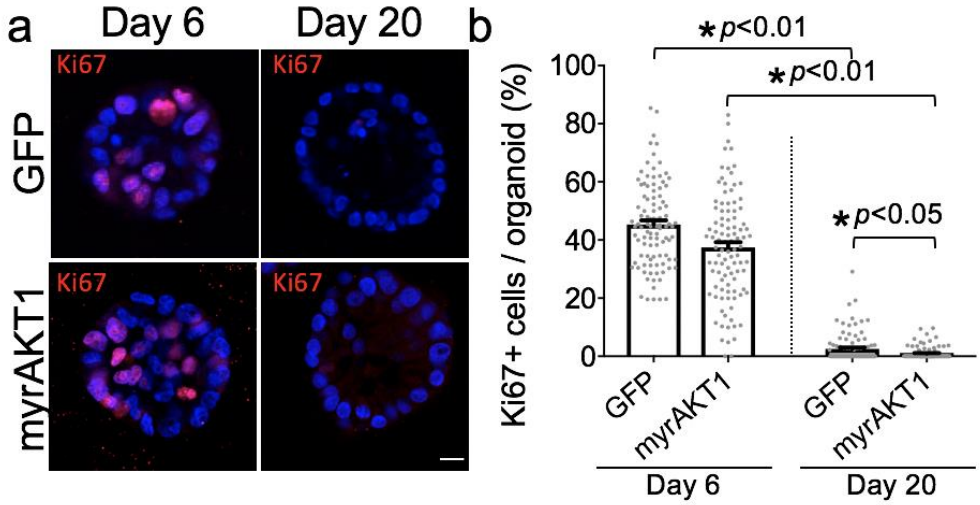

Figure 4: Organoid proliferation images and quantification of MCF10A myrAKT1 cells.

a) *Representative images and* ***b)*** *quantification of proliferating (Day 6) and growth-arrested (Day 20) organoids 2 days after doxycycline induction. Graphs indicate means and SEM of 100 organoids from three individual experiments. Statistical significant differences were determined by Student's t-test.*

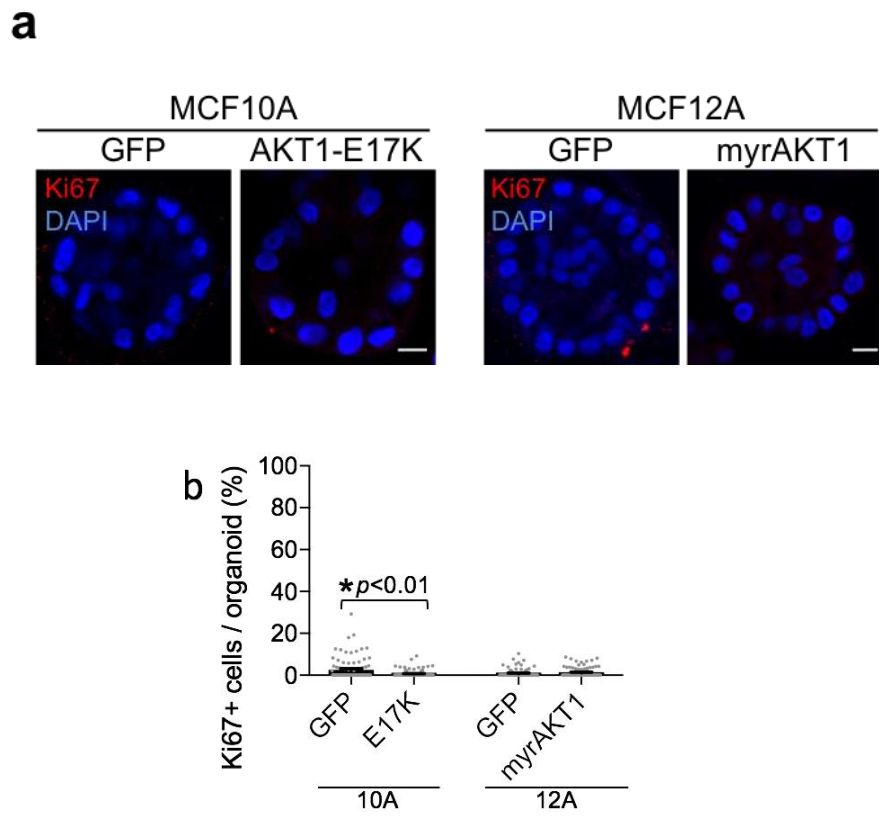

Figure 5: Organoid proliferation of MCF10A AKT-E17K and MCF12A myrAKT1 cells.

a) *Representative images and* ***b)*** *quantification of organoids of Day 20, four days after doxycycline induction of GFP or AKT activation. Two cell models, MCF10A cells with over-expression of the activating AKT1 mutant E17K found in breast cancer patients (MCF10A/AKT1-E17K) and MCF12A cells with myrAKT1 (MCF12A/myrAKT1). Cells remain largely growth arrest after induction of AKT signaling in both models. Graphs indicate means and SEM of 100 organoids from three individual experiments. Statistical significant differences were determined by Student's t-test. Scale bars, 10μm.*

Chapter 4: Quiescence Maintenance via Cell-Intrinsic Feedback Signaling

4.1 Introduction

A critical stage in early breast tumorigenesis is the emergence of preneoplastic cells that carry key tumor drivers but remain in a latent state similar to normal quiescent cells[71,74]. Such latent preneoplastic cells have been reported in precancerous lesions and tissues surrounding tumors, and are implicated as precursors for primary tumors[71]. Some of these preneoplastic breast lesions include flat epithelial atypia (FEA), atypical ductal hyperplasia (ADH), and lobular neoplasia in situ (LIN)[59]. Each of these lesions looks phenotypically normal but contains cells that are genetically altered and carry oncogenic drivers. Although oncogenic drivers are present, these lesions are not considered malignant and the proliferation rates of the cells within these lesions are very low (2-7%) suggesting that genetically altered cells carrying oncogenic changes are not drivers of proliferation as they remain in a quiescent state[68,73].

Evidence has shown that quiescence does not reflect a longer G1 phase within the cell-cycle, but rather a distinct state[63]. A hallmark of dormancy is that it has the capacity to reverse growth-arrest and enter back into the cell cycle[46]. Cells enter quiescence in response to an absence of growth factors or situational cues. Whether a preneoplastic quiescent cell re-enters the cell cycle and evolves

into a proliferating cancer cell depends on environmental cues- this progression is not necessarily only intrinsic. Understanding and identifying the distinguishing characteristics and signaling mechanisms that underlie the induction and maintenance of preneoplastic quiescence from proliferating preneoplastic cells will provide insights into therapeutic strategies to delay or intervene with the development of tumors[30,63].

Preneoplastic cells can perplexingly stay latent and resemble normal quiescent cells, despite carrying oncogenic drivers [46,63]. Sustained growth-arrest of these preneoplastic cells raises questions of the control that underlies their advantage to clonally expand during premalignant development. Taking advantage of the previously mentioned model system to gain mechanistic insights into how preneoplastic mammary cells that possess growth-promoting alterations stay quiescent in a premalignant tissue environment, I investigated the quiescent maintenance in preneoplastic breast cells with aberrant AKT signaling, a major tumor driver found in early lesions of the breast and many other epithelial tissues[13,16].

Quiescent cells and actively proliferating cells express different gene expression patterns and have different functional phenotypes[47,49]. Since these genetically altered cells are not proliferating, I hypothesized that the cell-cycle machinery may differ between preneoplastic and control quiescent cells. Exploring the cell-cycle machinery, results showed a distinctly different cell-cycle regulation in neoplastic quiescent cells that implicates an altered homeostatic

state of quiescence that may respond differently to growth stimuli. The up-regulation of some cell-cycle machinery was altered in cells with aberrant AKT activation along with other anti-proliferative proteins. This up-regulation suggests that although aberrant AKT activation is known as a driver of proliferation and growth, the pro-growth signal also induces cell-intrinsic feedback signaling mechanisms as a constraint signal to maintain a quiescent state.

Looking further into genetic expression differences of preneoplastic quiescent cells when compared to control cells, results showed different genetic expression profiles between preneoplastic, quiescent cells and control, quiescent cells other than cell-cycle machinery. The major up-regulated gene being biglycan (BGN) in preneoplastic, quiescent cells compared to the control cells. BGN is a small leucine-rich repeat proteoglycan (SLRP) found in a variety of extracellular matrix tissues[60]. BGN is expressed ubiquitously and is synthesized as a precursor from which an N-terminal pro-peptide is cleaved off by bone morphogeneic protein (BMP1) to yield the mature form[60]. When BGN is secreted, it interacts via its core protein or GAG chains with numerous components of the ECM, including Type I, II, III, and IV collagen and elastin, thereby becoming sequestered in the ECM of most organs[65]. This up-regulation of BGN in the preneoplastic, quiescent cells suggests that although aberrant AKT activation is present, this pro-growth signal also induces a cell-intrinsic feedback mechanism that alters the surrounding environment to help maintain quiescence.

4.2 Results

4.2.1 Cell-Cycle Machinery Expression Differs from Normal Cells

Given the predominant roles of AKT signaling in supporting cell proliferation and breast cancer development[33], I next investigated how quiescent preneoplastic cells with aberrant AKT activation maintain growth-arrest. I noticed that in two of the three models tested (Figure 5), the AKT-induced, preneoplastic cells have a statistically significant lower percent of $Ki67^+$ population (0.9±0.2% AKT1 and 0.6±0.2% E17K) compared to the already low percentage in the control populations (2.6±0.5%, Figures 4,5). These observations lead me to hypothesize that distinct homeostatic control of cell cycle sustains the growth-arrest of these preneoplastic cells. I focused on the CIP/KIP family of cyclin-dependent kinase inhibitors because MCF10A and MCF12A cells have a p16 loss[11], a common change in premalignant breast lesions[9]. A recent study has shown that p57Kip2, but not p21Cip1 nor p27Kip1, mediates insulin- and PI3K/AKT-mediated proliferation in mammary epithelial cells[34] promoting me to investigate the regulation of p57Kip2 in these preneoplastic cells. I found that both mRNA transcript and protein levels of p57Kip2 are significantly increased in the quiescent preneoplastic cells, compared to the controls (Figures 6 and 7). To determine the functional role of this p57Kip2 upregulation, we examined the effects of attenuating this p57Kip2 upregulation on the growth-arrest state of the

quiescent preneoplastic cells. I found that knockdown of p57Kip2 with two individual shRNA clones in the quiescent preneoplastic cells is sufficient to drive cell cycle re-entry (Figure 8). Together, these data demonstrated a crucial role of p57Kip2 in the maintenance of growth-arrest in preneoplastic cells with aberrant AKT signaling and highlight the distinct cell cycle regulation in these cells.

4.2.2 Genetic Expression Differs Between Proliferating and Quiescent Cells

To explore the genetic expression difference between preneoplastic quiescent and normal quiescent cells, I used the same three-dimensional model with aberrant AKT active cells to represent quiescent, preneoplastic breast cells. I grew both the preneoplastic and control cells to Day 20 and collected RNA for a microarray, additionally I collected RNA from both the control and preneoplastic cells on Day 6 to represent a proliferating state. The microarray was able to compare genetic differences between quiescent control cells and quiescent preneoplastic cells, while eliminating normal proliferating to quiescent genetic changes using RNA from the proliferating state. Only a handful of genes were significantly up-regulated or down-regulated in the preneoplastic quiescent cells when compared to the control cells. Representing this data in a heatmap (Figure 9), it is clear that most genetic changes were down-regulations in the quiescent preneoplastic cells when compared to control cells, although there were a few up-regulation changes in the quiescent preneoplastic cells. The most significant

change was an up-regulation in the gene encoded for the biglycan protein (BGN). The BGN gene was expressed at significantly higher levels in the quiescent AKT-activated cells than in the control quiescent cells. Furthermore, AKT quiescent cells also expressed this gene at significantly higher levels than in the AKT-activated proliferating cells. Re-collecting RNA from Day 6 and Day 20 for both control and quiescent cells, I performed a qPCR to confirm the microarray results which showed the same trend of a higher expression of BGN RNA in quiescent preneoplastic cells than proliferating preneoplastic cells or control cells (Figure 10).

Many studies have tried to elucidate the biological role of BGN. Recent studies have shown evidence suggesting that BGN may also act as a signaling molecule in addition to acting as a structural component of the ECM [55,60]. It is firmly established that BGN is part of the innate immune system and plays a crucial role in the regulation of inflammation[70]. New findings indicate that only un-sequestered BGN is capable of acting as a signaling molecule at least in inflammation[66,70]. Therefore, the amount of BGN in tissue sections does not necessarily reflect its biological effect as it represents mainly BGN that has been sequestered in the ECM[70].

Reflecting its widespread expression and complex function, it has been reported that BGN is involved in numerous experimental and human diseases[70], including cancer[50,55,58]. Numerous studies have exhibited that BGN has both promoting and inhibitory effects on tumor cells in various cancers[66-70]. BGN

expression has been found to be an indicator of poor prognosis in both colorectal and gastric cancer[67,69]. Conversely, the over-expression of BGN was reported to inhibit the growth of pancreatic cancer cells *in vitro*[68]. Additionally, HER-2/neu mediated oncogenic transformation, the malignant phenotype of these cells, was associated with the down-regulation of BGN[50].

4.2.3 BGN Over-Expression Decreases Cell Proliferation

Due to the contradictory roles BGN has been reported to have, the tumor progression role that BGN has likely depends on the stage, differentiation, and type of tumor. Therefore, to gain a better understanding of BGN in preneoplastic breast lesions, I assessed the role of BGN in quiescent, preneoplastic breast cells with aberrant AKT activity. Using MCF10A cells genetically altered to inducibly express aberrant AKT activity with an iCSZ reporter, we added another inducible vector to known-down BGN. With these AKT-expressing, BGN-knock-down cells, along with just AKT-expressing empty vector cells as a control, I plated them using the three-dimensional model system until Day 20. I then immune-stained these slides for Ki67 and BGN. As expected, BGN protein staining was higher in the control AKT-expressing cells and lower in the AKT-expressing cells with the BGN-knock-down vector. Since BGN is seen to be over-expressed in quiescent preneoplastic cells with aberrant AKT activity, I hypothesized that BGN plays a role in quiescent maintenance. Therefore, I

expected that knocking-down BGN would increase proliferation in these cells. This was the case, the BGN-knock-down cells had a significantly higher percent of proliferating cells (Ki67+) than the control cells (Figure 11), but this knock-down did not cause these cells to transform and grow colonies in soft agar (Figure 12). These results suggest that BGN is a cell-intrinsic signal to maintain quiescence in normal conditions and led me to hypothesize that over-expressing BGN in breast tumor cells may lessen growth. To explore this, I engineered T47D cells to constitutively over-express BGN and plated them in a soft agar assay comparing them to control T47D cells with an empty vector. This BGN over-expression vector did increase the expression of BGN (Figure 13). After 3 weeks, a noticeable difference in colony-formation was seen between the T47D BGN-over-expressing cells and the T47D control cells. The T47D control cells had more and bigger colonies than the T47D cells with BGN over-expression (Figure 13). These results suggest that BGN has an inhibitory effect in the breast tumorigenesis process.

4.3 Discussion

Taking advantage of the three-dimensional model system, I investigated quiescence maintenance in preneoplastic breast cells with aberrant AKT signaling. Overall, quiescent preneoplastic cells with aberrant AKT activation displayed a different genetic expression patterns than both quiescent normal

cells and proliferating neoplastic cells with aberrant AKT activation. These results highlight different functional phenotypes that quiescent preneoplastic cells displayed. I discovered that cell-intrinsic signaling played a role in quiescence maintenance. Furthermore, the genetic expression patterns of cell-cycle machinery were altered as well. An increase in p57Kip2 protein and mRNA expression, cyclin-dependent kinase inhibitor, was seen in cells with aberrant AKT signaling. Knocking-down p57Kip2 in these cells was sufficient to drive cell cycle re-entry suggesting that the cell-intrinsic up-regulation of p57Kip2 was to maintain a quiescent state.

Additionally, levels of BGN mRNA were significantly higher in quiescent, preneoplastic cells with aberrant AKT activation than both normal quiescent cells and proliferating preneoplastic cells with aberrant AKT activation. This suggests that BGN up-regulation also seems to play a role in quiescent maintenance, but since BGN is a secreted protein, BGN regulation of quiescent maintenance differs from p57Kip2. As BGN is secreted into the microenvironment, it deposits and creates a niche that promotes and favors quiescence. Therefore, the up-regulation of BGN regulates quiescence maintenance through modulating the microenvironment surrounding the cells. This suggests that if the microenvironment changes, the favor towards quiescence may be overthrown causes cells to clonally expand.

Furthering our understanding of similar mechanisms underlying quiescence could have broad implications in possibly preventing breast tumor

progression from the earliest points of mutation. Furthermore, p57Kip2 and BGN may be potential markers in preneoplastic lesions and could be developed further into possible diagnostic and risk assessment procedures.

4.4 Figures

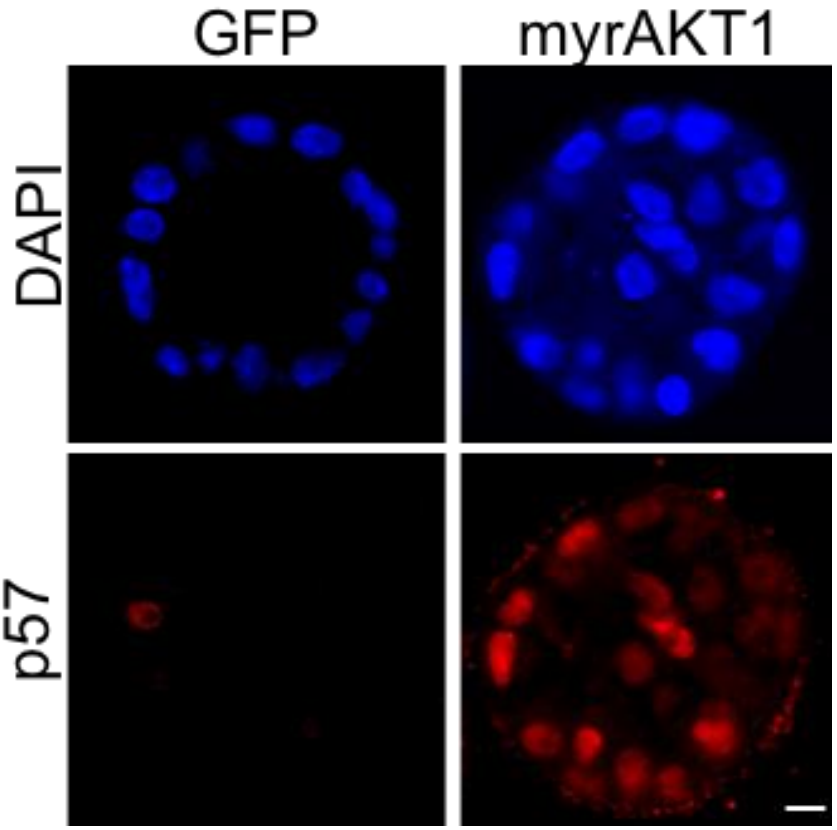

Figure 6: MCF10A myrAKT1 cells up-regulate p57Kip2 protein expression. Representative images of AKT induced and control quiescent organoids immunostained with p57Kip2.

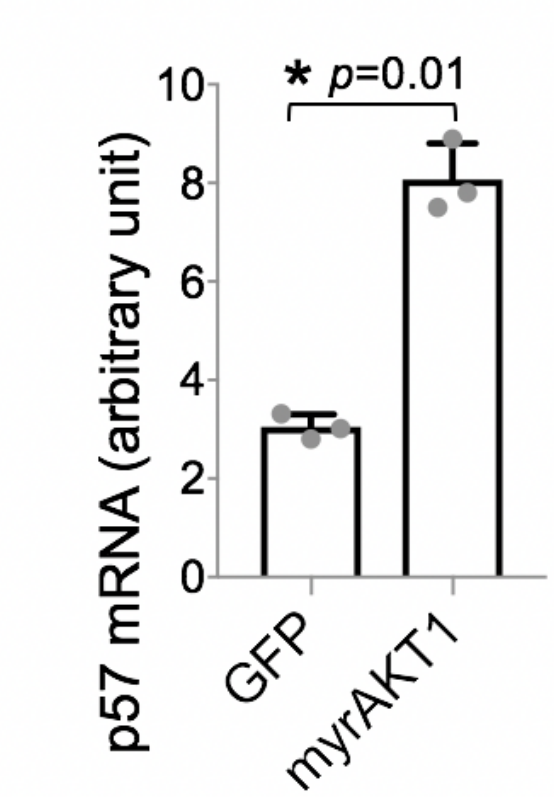

Figure 7: MCF10A myrAKT1 cells up-regulate p57Kip2 mRNA expression.

qPCR of p57Kip2 mRNA transcript expression normalized to RPLR0. The graph indicates means and standard deviations from three individual samples.

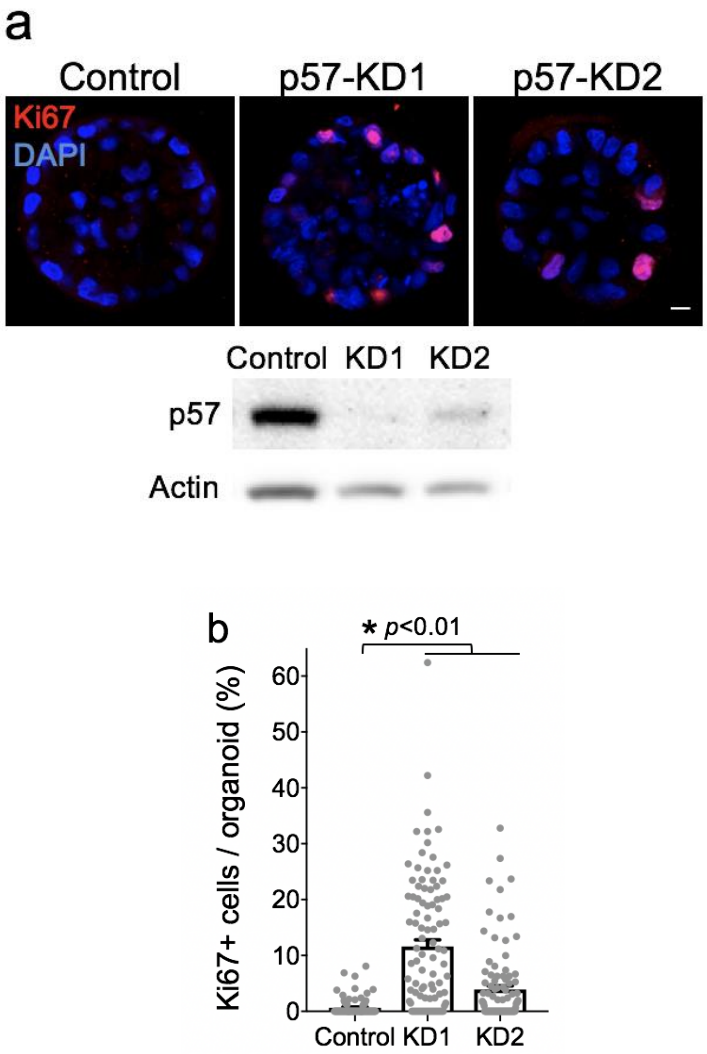

Figure 8: p57Kip2 knock-down promotes cell cycle re-entry of MCF10A myrAKT1 cells.

a) *Representative images and* ***b)*** *quantification of AKT-induced, preneoplastic cells with knowndown by two p57Kip2 shRNA and control. Graphs indicate means and SEM of 100 organoids from three individual experiments. Statistical significant differences were determined by Student's t-test. Scale bars, 10μm.*

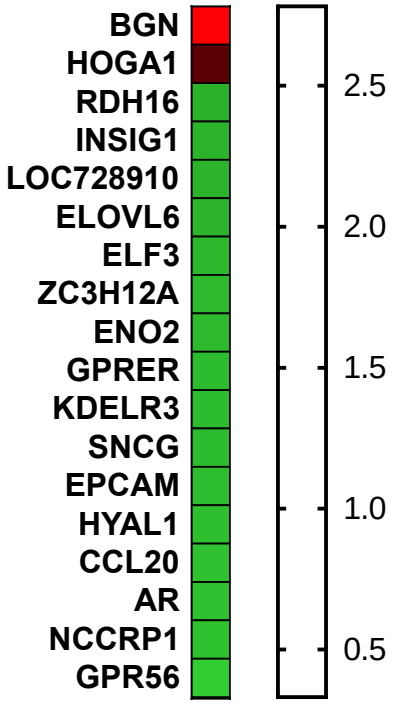

Figure 9: MCF10A myrAKT1 Quiescent vs. Proliferating mRNA Heatmap

Comparing myrAKT1 quiescent mRNA (Day 20) and myrAKT1 proliferating (Day 6) mRNA from MCF10A cells in 3D culture. This list only includes genes that are significantly changed by 2 folds or more.

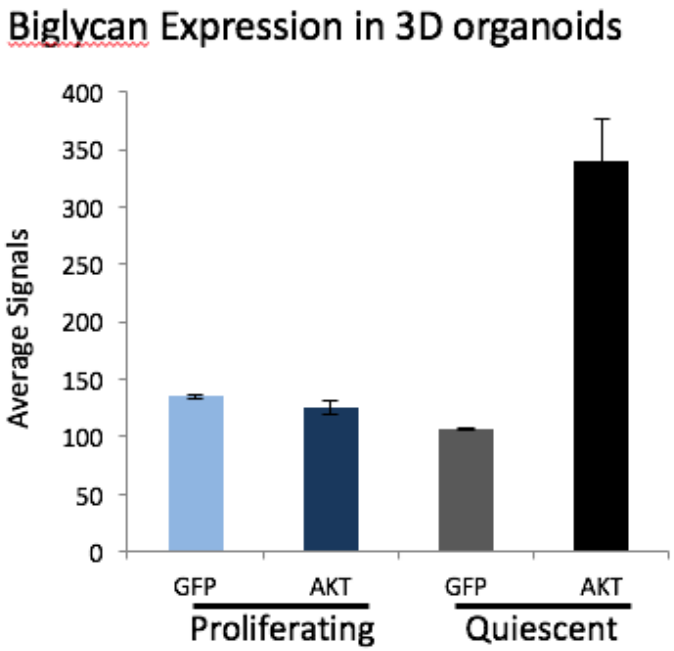

Figure 10: MCF10A myrAKT1 over-expresses BGN RNA

Graph shows mRNA levels of BGN in proliferating and quiescent MCF10A GFP Control and myrAKT1 cells at Day 6 and Day 20.

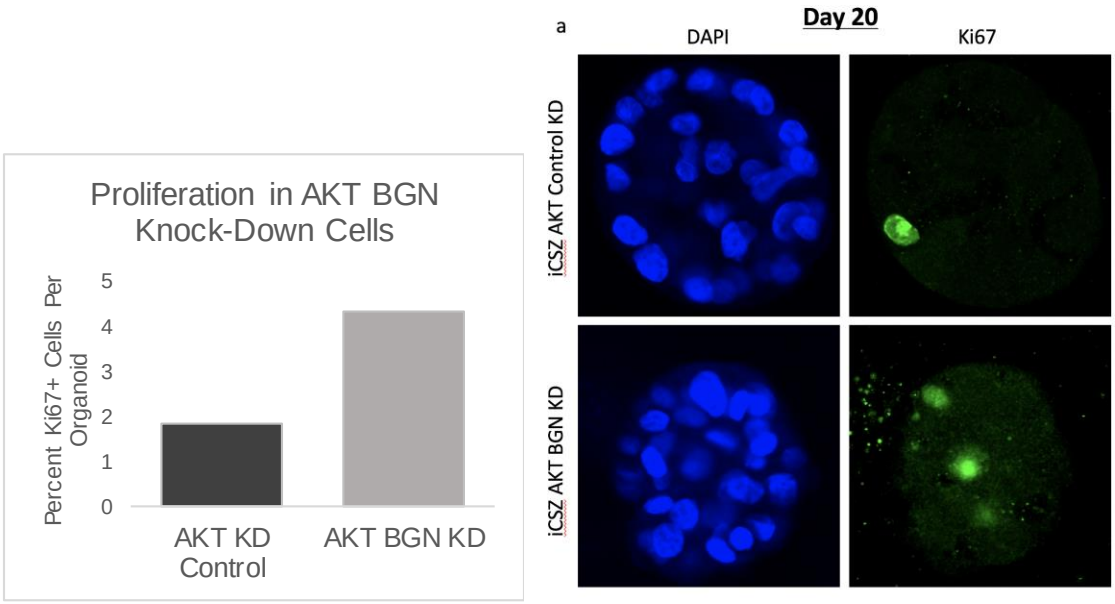

Figure 11: BGN knock-down increases proliferation in MCF10A myrAKT1 cells

a) *Representative images and* ***b)*** *quantification of AKT-induced, quiescent BGN-knock-down and control organoids stained for Ki67 at Day 20. BGN-knock-down promotes proliferation of the preneoplastic cells with aberrant AKT activation.*

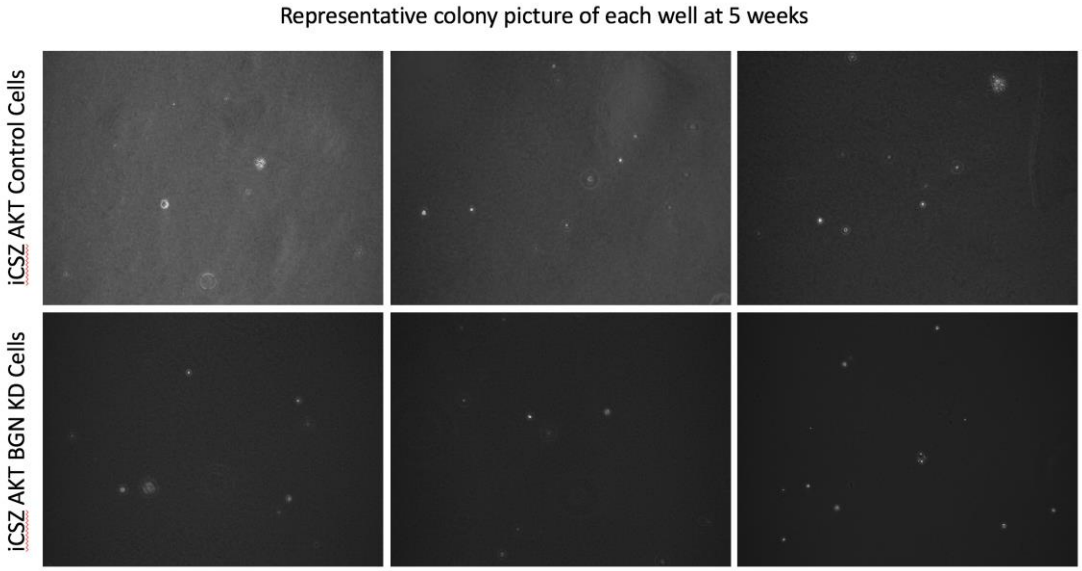

Figure 12: BGN knock-down does not promote cell transformation

Representative images (10x objective) of MCF10A cells with inducible myrAKT1-iCSZ with or without expression of a BGN-knock-down vector.

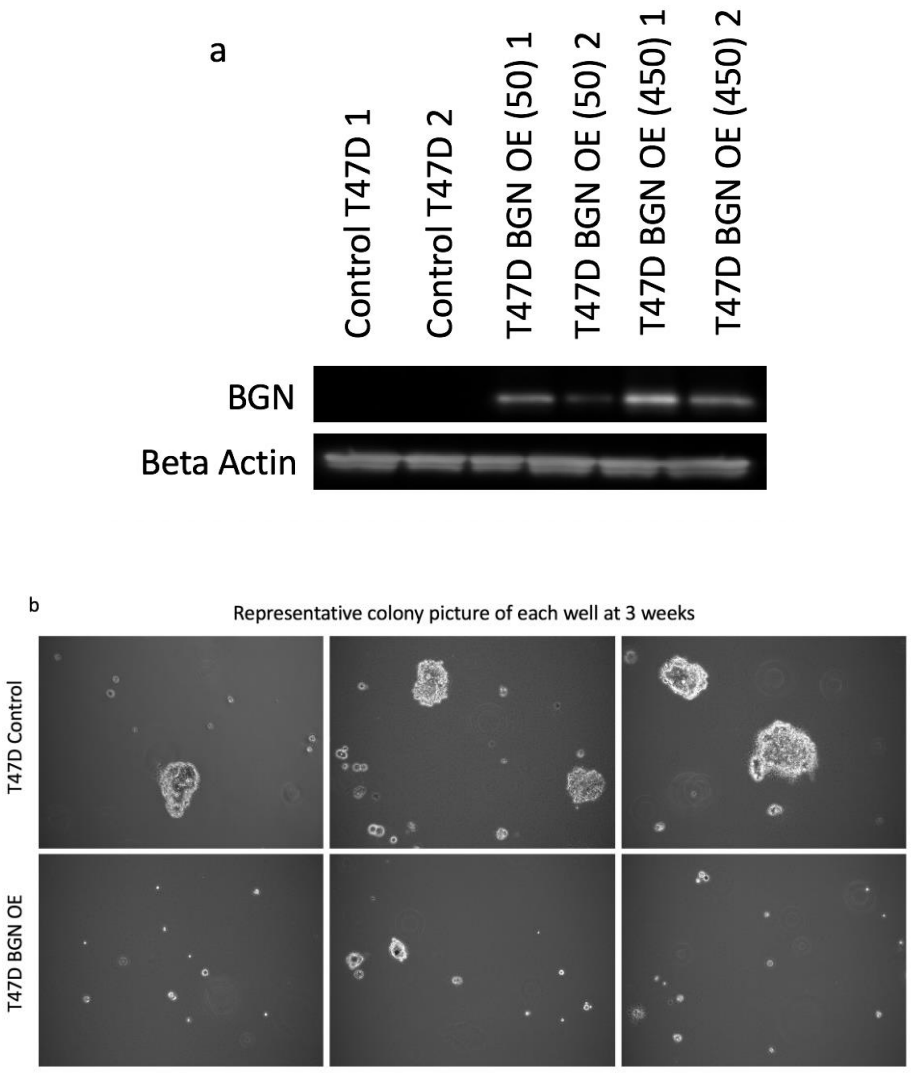

Figure 13: BGN over-expression inhibits growth of breast tumor cells

a) *Western blot verification of BGN over-expression.* ***b)*** *Representative pictures of soft agar assay of T47D cells over-expressing BGN.*

Chapter 5: Microenvironment Interactions Promotes Clonal Expansion Advantage

5.1 Introduction

A hallmark of dormancy is that has the capacity to reverse growth-arrest and enter back into the cell cycle[34]. This ability to exit from a quiescent state is the clinically most relevant phase[34,56]. The expansion of preneoplastic cells during premalignant development is fundamental to driving clonal evolution and tumorigenesis[1]. Preneoplastic cells can perplexingly stay latent and resemble normal quiescent cells, despite carrying oncogenic drivers. Controls underlying this preneoplastic clonal expansion remain largely elusive. Whether a preneoplastic quiescent cell re-enters the cell cycle and evolves into a proliferating cancer cell depends on environmental cues- this progression is not necessarily only intrinsic. Tumorigenic niches have important impacts on this progression. Therefore, better understanding preneoplastic, quiescent cells are required for elucidation of biochemical and genetic events involving clonal evolution during tumor development.

During the breast tumorigenesis process, preneoplastic cells and the stromal environment work hand-in-hand to actively drive tumor progression. Quiescent preneoplastic cells are dependent on their surrounding environment for cues on whether to remain latent or grow, therefore the stromal

microenvironment plays a critical role in tumor initiation and proliferation. Mammary epithelial cell growth is dependent on epidermal growth factors (EGF)[31]. EGF is a growth factor that stimulates cell growth, proliferation, and differentiation by binding to its receptor, EGFR[31]. Deregulation of EGF activation has been shown to support breast tumor development[31] and therefore I wanted to see whether this pathway was altered in the preneoplastic quiescent cells using the previously mentioned three-dimensional model system. Cells with aberrant AKT activation were grown until growth arrest and then exposed to different levels of EGF. Even with high levels of EGF-exposure, these cells remained in a quiescent state suggesting a homeostatic shift in response to growth stimuli. Therefore, these preneoplastic quiescent cells might be sensitive to growth stimuli present in the surrounding microenvironment.

The mechanisms that drive preneoplastic quiescent cells to re-enter the cell cycle remain unclear. This critical decision is tightly coupled with interactions between preneoplastic cells, host cells, and immune mechanisms. Specific immune effectors and secreted factors (including cytokines and chemokines) have been implicated in the initiation of tumorigenesis and tumor growth. Similarly, inflammation can be associated with increased tumorigenesis as chronic inflammation has been associated with a poorer breast cancer prognosis. Consequently, the presence or absence of immune effectors is associated with a favorable or non-favorable prognosis depending on tissue type, thereby highlight the complexity of the host immune system and evolving tumor interaction. This

suggests that immune cells are important to the pathogenesis of tumorigenesis[2-4]. Understanding the signaling mechanisms that underlie the induction and maintenance of preneoplastic quiescence will provide insights into therapeutic strategies to delay or intervene with the development of tumors.

Using a three-dimensional co-culture model system, previous studies have investigated the effects of both normal fibroblasts and Carcinoma-associated fibroblasts (CAFs) on preneoplastic breast cells. Results showed that normal mammary fibroblasts seemed to inhibit proliferation, while CAFs induced epithelial cell growth and morphogenesis. Therefore, balancing between tumor-promoting and tumor-suppressive effects of CAFs may hinge on the heterogeneity of CAF populations, as well as other components in the microenvironment. Altogether, breast CAFs support breast cancer proliferation via secretion of various growth factors and cytokines, but the mechanisms underlying the upregulation of these factors, downstream pathways, and the cross-talk between factors is not clear. Even further, it is unclear whether these secreted factors from breast CAF are the consequence of cancer or if initiated before cancer transformation. Despite the understanding of characteristics associated with breast CAFs, further investigation into their role and involvement of the breast cancer microenvironment will yield important and needed insights to the convoluted connections between cancer cells and the stroma.

Given the increasing importance of immune-oncology and the dramatic rise in the use of targeted therapy in the clinic, I investigated the role of the

immune system in clonal expansion in preneoplastic breast cells. Here, using organoid models of quiescent preneoplastic breast cells with aberrant AKT activation, I found that distinct molecular controls in the preneoplastic cells contribute to an altered homeostatic state of quiescence, which underlies the maintenance of growth-arrest under normal conditions and the proliferative advantage in suppressive tissue environments. These quiescent preneoplastic cells express increased levels of the cycling-dependent kinase inhibitor p57Kip2 to sustain growth-arrest, despite AKT hyperactivation, as previously mentioned. Concurrently these cells show an upregulation of CXCR4 expression that confers the cells competence to proliferate in response to CXCL12, a chemokine implicated in cancer-associated stroma. CXCL12 promotes proliferation through ERK and PKA, does not induce neoplastic transformation, but suffices to drive clonal expansion in suppressive mammary organoids. Our studies propose a model in which an oncogene-mediated switch of mitogen usage promotes selective preneoplastic clonal expansion in permissible microenvironments.

5.2 Results

5.2.1 EGF is not Sufficient to Promote Outgrowth

Mammary epithelial cell growth is dependent on epidermal growth factors (EGF)[31]. EGF is a growth factor that stimulates cell growth, proliferation, and differentiation by binding to its receptor, EGFR[31]. Deregulated EGF activation has been shown to support breast tumor development[31]. I first tested whether quiescent preneoplastic cells are prone to proliferate in elevated levels of exogenous EGF. I exposed the growth-arrested organoid cells with aberrant AKT activation or controls to media with increasing EGF levels up to four times (20ng/mL) the original condition (Figure 14), a concentration significantly higher than the reported serum EGF concentration (~0.6ng/ml) [32]. Surprisingly, both the control and the AKT-induced, preneoplastic cells remained largely quiescent, suggesting that these quiescent preneoplastic cells are no longer sensitive to EGF-induced proliferation.

5.2.2 CAFs Promote Cell-Cycle Re-Entry

I hypothesized that quiescent preneoplastic cells are more prone to proliferate in stromal environments associated with increased cancer risk secreted factors. Premalignant cells in tissue fields surrounding resected tumors have been implicated in the development of second or recurrent tumors[35,36]. CAFs are key components of breast tumor-associated stroma that often persist

with residual preneopalstic cells after tumor resection and have been shown to secrete factors that contribute to tumor development[37]. I therefore took advantage of an established CAF model, cavioln-1 knockout (Cav1-KO) NIH3T3 cells, with a secretome similar to human breast CAFs[38,39], to test my hypothesis and explore the proliferative control of quiescent preneoplastic cells. I exposed AKT-induced, quiescent preneoplastic cells to conditioned-media (CM) from CAFs or control fibroblasts, and determined their proliferative responses (Figure 15 A). I found that exposure to CM from Cav1-KO fibroblasts significantly increased the percent of proliferating cells (Ki67+) in the quiescent preneoplastic organoids (Figure 15 B, C), suggesting that stromal environments with CAFs may favor clonal expansion of quiescent preneoplastic cells.

5.2.3 CXCL12 Signaling Drives CAF-Promoted Cell-Cycle Re-Entry

To gain mechanistic insights into this CAF-induced proliferation, I identified secreted factors upregulated in the Cav1-KO fibroblasts using quantitative mass spectroscopy. I focused on CAF-derived chemokines because they have been implicated in mediating stromal-epithelial crosstalk[36,39]. I found that three chemokines, CXCL1, CXCL7, and CXCL12, are significantly more abundant in the Cav1-KO CM than the control (Figure 16 A). To determine their role in the observed phenotypes, I examined the proliferative response of these quiescent preneoplastic cells when systematically exposed to the mentioned

chemokines individually. I found that recombinant CXCL12, but not CXCL1 nor CXCL7, drives a subtle but significant increase in the percent of proliferating cells per organoid (Figure 16 B, C). Importantly, previous studies have shown that CXCL12 is upregulated in carcinoma-associated fibroblasts from breast and pancreatic cancer patients[36,40]. The mechanisms underlying the small percentage of cells that exit quiescence and proliferate in response to CXCL12 is unclear, but may reflect additional homeostatic controls that restrain proliferation of preneoplastic cells and suppress tumorigenesis during early development.

Previous studies have demonstrated that CXCL12 plays a critical role in promoting malignant cell growth, angiogenesis, and metastasis in advanced stages of tumorigenesis[36,43,44], but its contribution to premalignant development is unclear. I further investigated the CXCL12-mediated signaling crosstalk to the quiescent preneoplastic cells. I found that CXCL12 selectively drives proliferation of the AKT-induced, quiescent preneoplastic cells, but not the control quiescent cells (Figure 17). This CXCL12-induced selective proliferation was also observed in MCF12A cells overexpressing myrAKT1 and MCF10A cells overexpressing AKT1-E17K (Figure 18). Notably, in addition to CAFs, upregulation of CXCL12 is also evident in other cellular conditions associated with increased cancer risk, such as aging (senesced fibroblasts) [41] and obesity-induced inflammatory tissue environments[42]. Together, these data demonstrated a role of stromal conditions in promoting selective proliferative outgrowth of preneoplastic cells and identified CXCL12 as a key factor mediating this selective proliferative response.

5.2.4 CXCL12 Promotes Cell-Cycle Re-Entry Through PKA and MEK via CXCR4

To elucidate the molecular basis underlying this selective proliferation response, I examined the expression of two CXCL12 receptors, CXCR4 and CXCR7[45], in the quiescent preneoplastic cells. I found that mRNA transcription of CXCR4 is increased in the AKT-induced, quiescent preneoplastic cells compared to the control quiescent cells, which CXCR7 mRNA transcripts were undetectable by qPCR (Figure 19 A, B). Immunofluorescent staining also showed an increase in the abundance of CXCR4 proteins on the cell membrane of quiescent preneoplastic cells with aberrant AKT activation (Figure 19 C). Functionally, I found that inhibiting CXCR4 with AMD3100, specific CXCR4 antagonist, significantly blocked the CXCL12-induced proliferation (Figure 20). Similar effects were found in MCF10A cells with inducible AKT1-E17K and MCF12A cells with activated myrAKT1 (Figure 21). AMD3100 also blocked proliferation induced by the CM from Cav1-KO fibroblasts (Figure 22). Moreover, I examined the signaling downstream of CXCR4 in mediating the CXCL12-induced proliferation. Treatment with inhibitors of MEK (PD325901) and PKA (H-89), but not PKC (GF109203X) (Figure 23), blocked the proliferative outgrowth. These data together suggest that CXCL12 induced proliferation of preneoplastic cells through CXCR4, MEK, and PKA signaling. Furthermore, I determined whether this CXCR4-mediated proliferation requires continual aberrant activation of AKT signaling. Cell lines were generated with an inducible CXCR4 expression cassette and I found that overexpressing CXCR4 alone, without overexpressing

myrAKT1, in quiescent organoid cells is sufficient to confer proliferative response to exogenous CXCL12 (Figure 24). Altogether, these data suggest that the AKT-induced upregulation of CXCR4 during growth-arrest underlies the selective competence of the quiescent preneoplastic cells to proliferate in response to exogenous CXCL12 derived from CAFs or other tumor-promoting microenvironments.

Successful clonal expansion depends on not only cell proliferation but also survival of the progeny. To gain biological insights into the CXCL12-induced proliferation, I determine its contribution to successful clonal expansion of preneoplastic cells in the premalignant organoids. I transduced single-cells with low doses of myrAKT1-IRES-GFP, or IRES-GFP control, lentivectors in growth-arrested mammary organoids and determined the clonal expansion of transduced cells after exposing the organoids to CXCL12. Single cells transduced with myrAKT1 or GFP control remain largely as single-cells under normal growth conditions, consistent with the maintenance of quiescence in preneoplastic cells (Figure 25 A). CXCL12 treatment significantly promoted clonal expansion of transduced preneopalstic cells with aberrant AKT activation, but not in the GFP control (Figure 25 B). Interestingly, CXCL12 treatment did not drive anchorage-independent growth of either AKT-induced preneoplastic cells and control cells on soft agar (Figure 26). These data support that CXCL12 promotes selective preneoplastic clonal expansion in premalignant tissue prior to neoplastic transformation.

5.2.5 Increased CXCR4 Expression Favors Breast Tumor Initiation and Progression

Lastly, my findings of CXCR4 signaling in promoting preneoplastic clonal expansion predicts that elevated CXCR4 expression level favors breast tumor initiation and progression. Consistent with this notion, quarry of patient sample databases (Oncomine™) show a significant increase in CXCR4 transcription in early stage breast ductal carcinoma *in situ* (3.0 folds, p=8.43E-7) and invasive carcinoma (2.2 folds, p=7.04E-28) compared to normal breast tissues (Figure 27).

5.3 Discussion

A hallmark of dormancy is the capacity to reverse growth-arrest and enter back into the cell cycle. This ability to exit from a quiescent state is the clinically most relevant phase as the expansion of preneoplastic cells during premalignant development is fundamental to driving clonal evolution and tumorigenesis. Although I gained more understanding into the cell-intrinsic mechanisms that help maintain quiescence by altering the genetic expression within the cells and how these mechanisms can modulate the environment to promote quiescence, it is unclear what triggers preneoplastic cells with pro-growth, oncogenic alterations to surpass this maintenance and re-enter the cell cycle. Whether a preneoplastic quiescent cell re-enters the cell cycle and evolves into a proliferating cancer cell may not necessarily be only intrinsic, but can depend on environmental cues. Tumorigenic niches have important impacts on this progression. Therefore, a better understanding preneoplastic, quiescent cells are required for elucidation of biochemical and genetic events involving clonal evolution during tumor development.

Using the previously mentioned reconstructed quiescent preneoplastic model with aberrant AKT activation, I elucidated the control of indolent preneoplastic cells during premalignant development. Exposing these aberrant AKT quiescent cells to high levels of EGF, there was no growth suggesting that these cells are insensitive to their native growth factor and assume an altered

homoestatic state that upregulates p57Kip2 and BGN secretion to sustain growth-arrest in normal conditions, while concurrently inducing the expression of CXCR4. Although EGF is present in the organoid cultures, the AKT-induced quiescent preneoplastic cells appear to be insensitive to EGF-stimulated proliferation, prompting me to investigate the growth regulation in these preneopalstic cells. Since cytokines and chemokines are known to be present surrounding preneoplastic lesions and breast tumors secreted from fibroblasts and CAFs, looking at the secretome for possible growth factors that promote proliferation of the quiescent, preneoplastic cells in replacement of EGF. Results showed a high secretion of CXCL12 that cells with aberrant AKT activation were selectively sensitive to compared to control cells. Additionally, an up-regulation of receptor CXCR4 protein and mRNA, who's major ligand is CXCL12, was seen in preneoplastic quiescent cells as well. This CXCR4 up-regulation confers the quiescent cells to selective capacity to respond to CXCL12. CXCL12 acts as an alternate mitogen derived from tumor-associated stroma, which promotes quiescent preneoplastic cells to proliferate and drives selective clonal expansion in a suppressive epithelial environment, ultimately replacing the native role of EGF. Furthermore, with the use of inhibitor studies, results show that the CXCL12-CXCR4 axis was signaling through PKA, MEK, and ERK in the preneoplastic, quiescent cells.

Altered homeostatic control of quiescence has emerged as a mechanism in muscle stem cells and resting T-cells that underlie normal physiological

functions critical for muscle repair and immune response[47,48]. My findings suggest that altered homeostatic controls of quiescence may also underlie the maintenance and selective clonal expansion of indolent preneoplastic cells during premalignant development. I demonstrated, in my model, an altered homeostatic control of quiescence mediated by aberrant AKT signaling and a mechanism of selective clonal expansion driven by a CAF-derived chemokine in a premalignant tissue environment. These specific findings seem to be selective for quiescent cells with an altered AKT pathway rather than universal for all preneoplastic quiescent cells with different oncogenic alterations. The specific homeostatic mechanisms will likely depend on the underlying oncogenic changes and tissue origins of the preneoplastic cells.

5.4 Figures

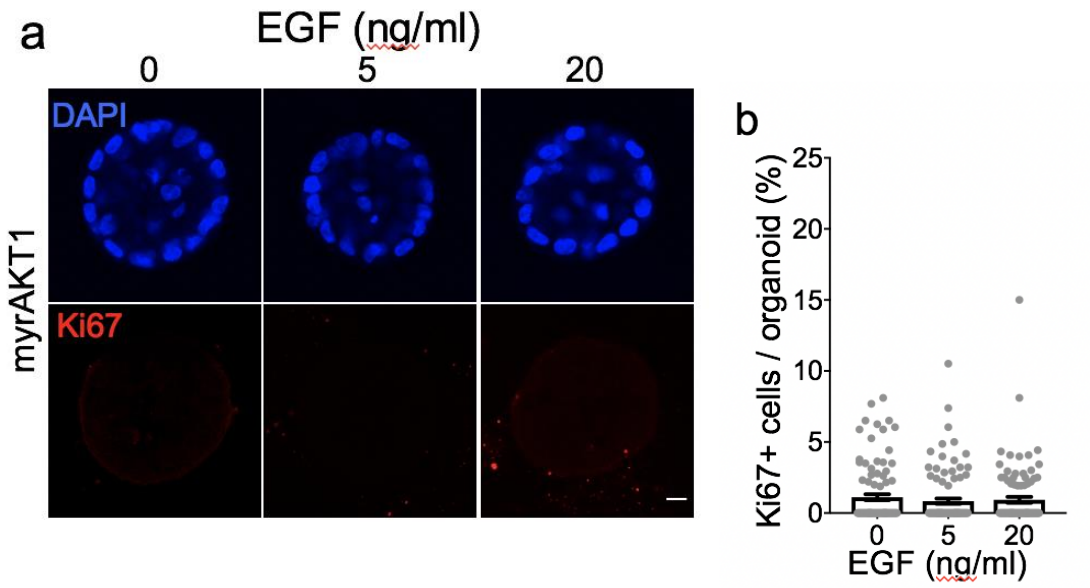

Figure 14: Cells remain growth-arrest despite different EGF concentrations.

a) *Representative images and* ***b)*** *quantification of GFP and myrAKT/GFP over-expressing organoids at Day 20 culture two days after doxycycline induction at different EGF concentrations. Cells remain growth-arrest.*

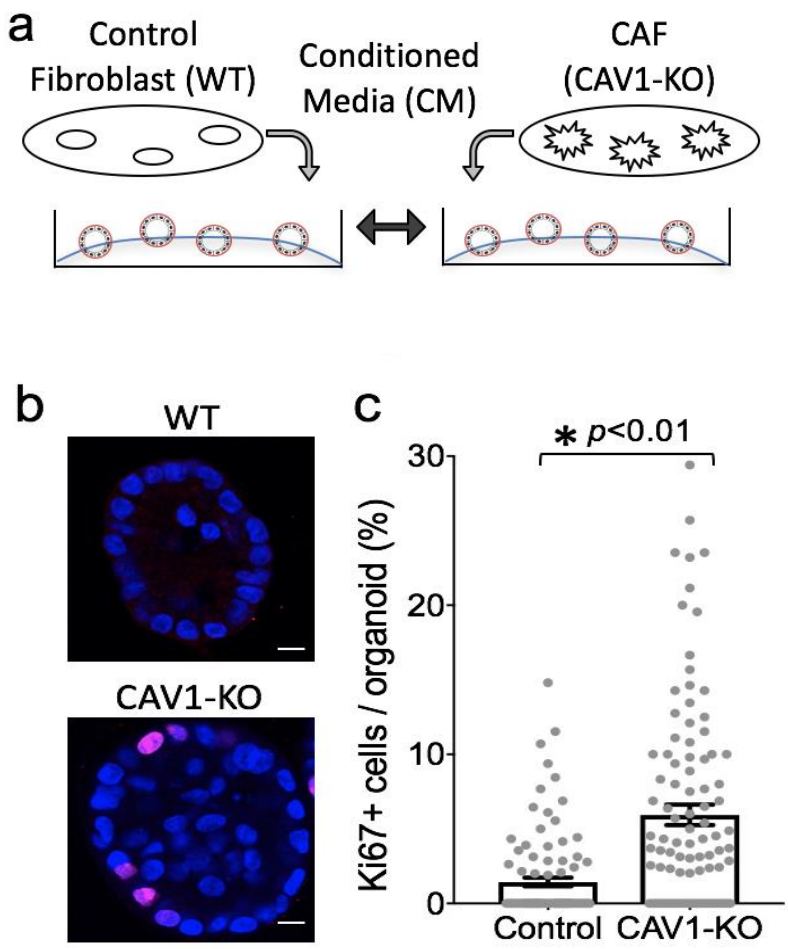

Figure 6: CAF conditioned media promotes outgrowth of MCF10A myrAKT1 cells

a) *Schematic of organoid treatment with conditioned media from carcinoma-associated fibroblast models.* ***b)*** *Representative images and* ***c)*** *quantification of AKT-induced premalignant organoids treated with conditioned media from WT or CAV1-KO fibroblasts.*

a

Protein Name	Uniprot ID	Sequence Coverage (%)	Normalized Ratio KO/WT	%CV	P-value
CXCL1	P12850	46	2.73:1	10	0.001
CXCL7	Q9EQI5	11	4.88:1	50	0.02
CXCL12	P40224	16	2.75:1	9	0.003

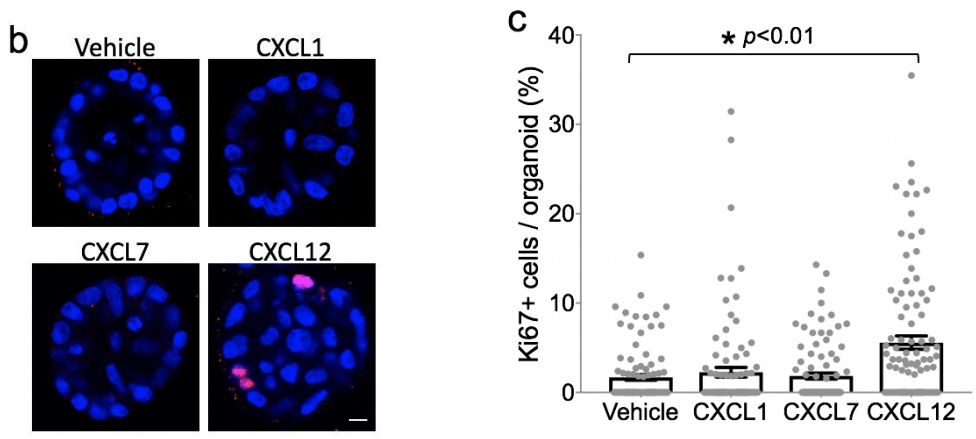

Figure 7: CAF-secreted CXCL12 mediates outgrowth of MCF10A myrAKT1 cells

a) *Up-regulation of chemokines in CAV1-KO fibroblasts. Proteins in serum-free conditioned meda from Cav1-KO and control fibroblasts were analyzed via LC/MS/MS. Three chemokines CXCL1, CXCL7, and CXCL12 were up-regulated more than two folds in the CAV1-KO conditioned media.* ***b)*** *Representative images and* ***c)*** *quantifications of AKT-induced premalignant organoids treated with 50ng/ml of CXCL1, CXCL7, CXCL12, or vehicle. Scale bar, 10μm. Graphs indicate means and SEM of 100 organoids from three individual experiments. Round dots represent individual data points. Statistically significant differences were determined by two-tail, Student's t-test.*

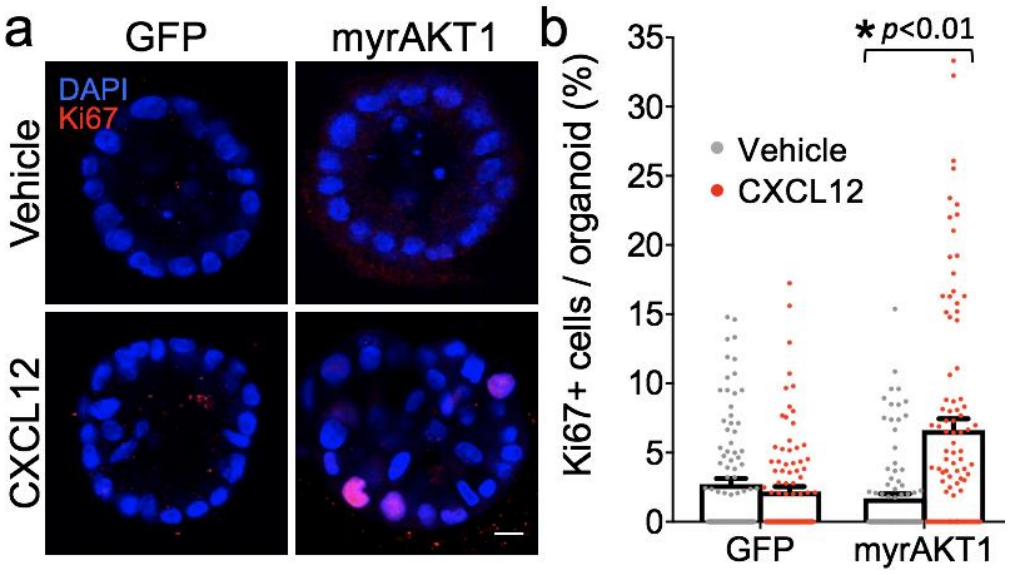

Figure 8: Recombinant CXCL12 promotes proliferation of MCF10A myrAKT1 cells

***a**) Representative images and **b**) quantification of AKT-induced, quiescent premalignant and control organoids treated with recombinant human CXCL12 or vehicle control. CXCL12 selectively promotes proliferation of the preneoplastic cells with aberrant AKT activation.*

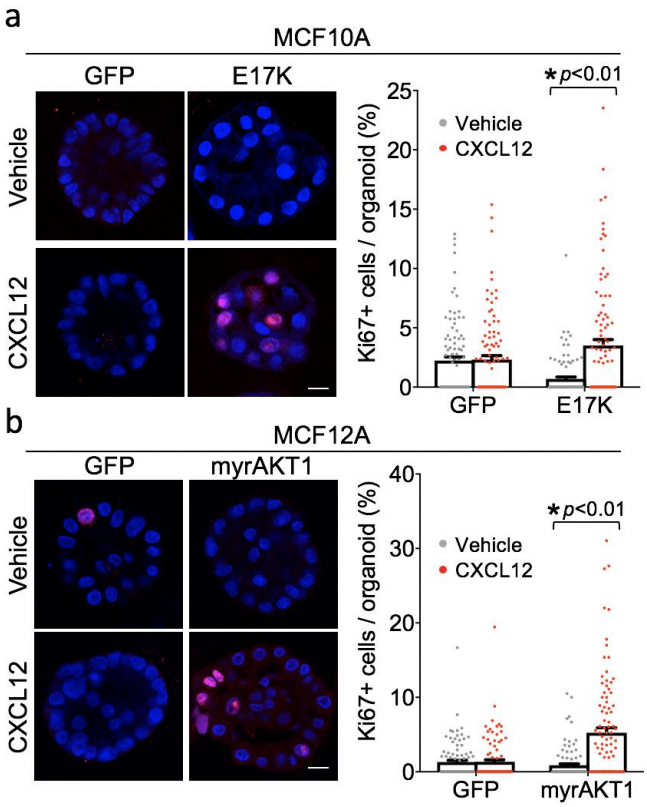

Figure 9: Recombinant CXCL12 selectively promotes outgrowth in AKT mutated cells

*Quiescent organoids derived from **a**) MCF10A/AKT-E17K or MCF10A/GFP control and **b**) MCF12A/myrAKT1 or MCF12A/GFP control were treated with recombinant CXCL12 or vehicle. Representative images and quantification of proliferative cells after treatment are shown. CXCL12 promotes proliferative outgrowth in both quiescent preneoplastic cell models with aberrant AKT activation. Graphs indicate means and SEM of 100 organoids from three individual experiments. Round dots represent individual data points. Statistically significant differences were determined by Student's t-test. Scale bars, 10µm.*

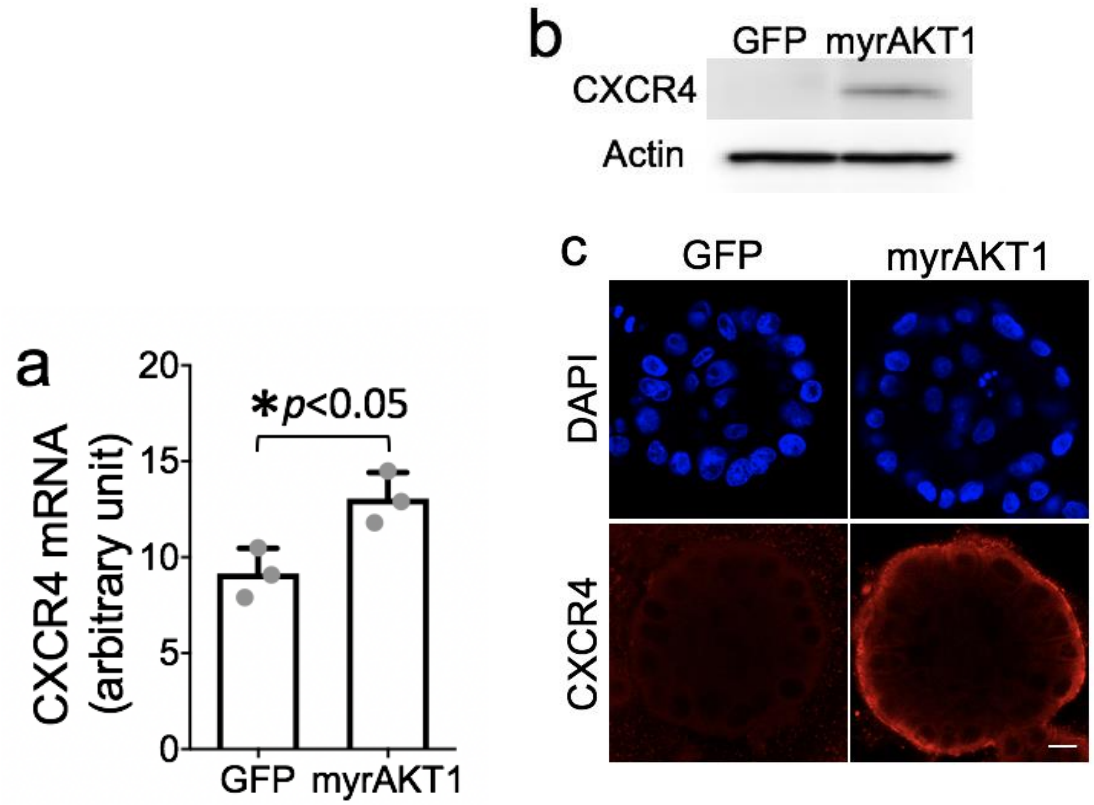

Figure 10: MCF10A myrAKT1 cells up-regulate CXCR4 protein and mRNA.

***a**) qPCR (mRNA level normalized to RPLPO), **b**) Western blots (protein), and **c**) immunostaining of CXCR4 in quiescent preneoplastic cells with aberrant AKT activation and control quiescent cells.*

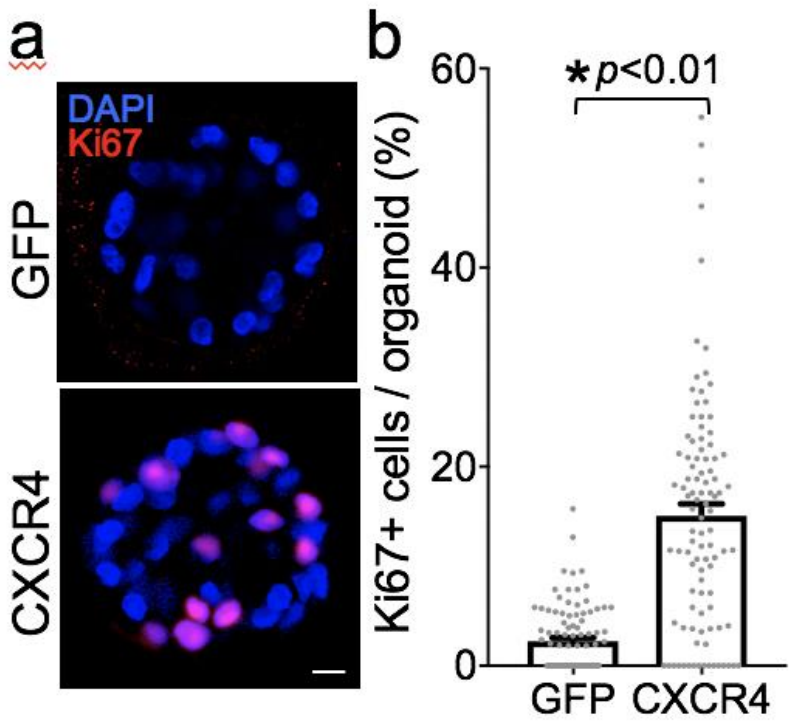

Figure 11: AMD3100 prevents CXCL12-mediated outgrowth.

***a**) Representative images and **b**) quantification of AKT-induced, quiescent preneoplastic cells treated with CXCL12 or CAV-1 KO CM in the presence of the specific CXCR4 antagonist AMD3100 or vehicle (PBS).*

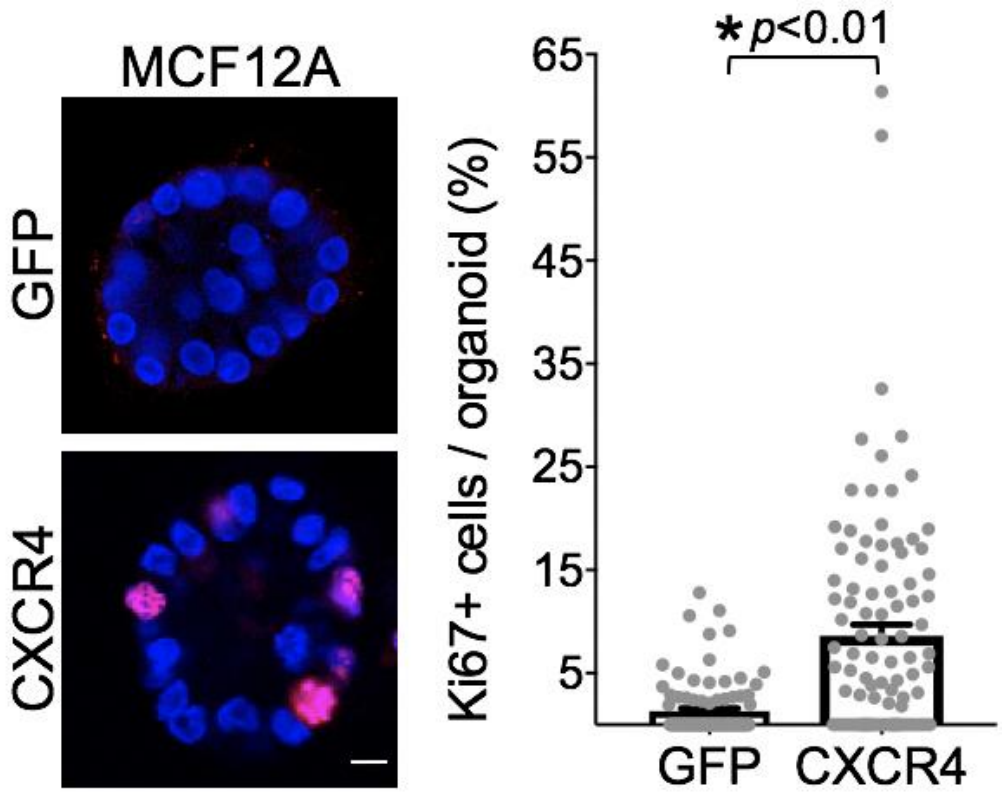

Figure 12: CXCR4 over-expression in control cells promotes outgrowth

Representative images and quantification of cell proliferation from growth-arrested MCF12A organoids induced to overexpress CXCR4-IRES-GFP or IRES-GFP control in media containing recombinant human CXCL12. CXCR4 overexpression is sufficient to confer sensitivity to exogenous CXCL12. The graph indicates means and SEMs of 100 organoids from three individual experiments. Round dots represent individual data points. Statistically significant differences were determined by Student's t-test. Scale bars, 10µm.

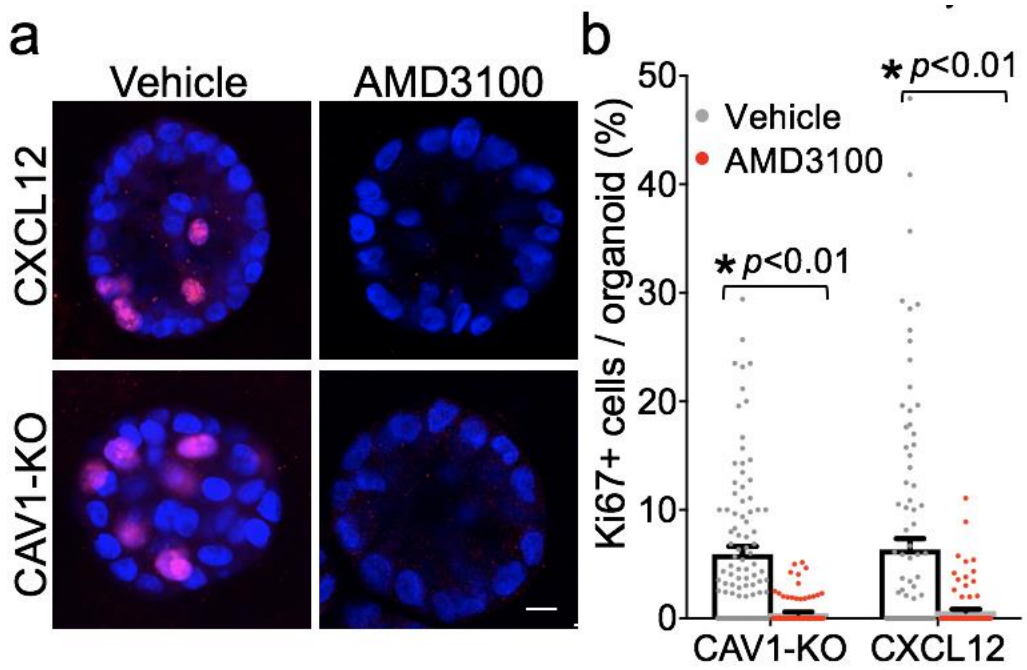

Figure 13: AMD3100 blocks CAF-mediated outgrowth of MCF10A myrAKT1 cells.

***a**) Representative images and **b**) quantification of AKT-induced, quiescent preneoplastic cells treated with CXCL12 or CAV-1 KO CM in the presence of the specific CXCR4 antagonist AMD3100 or vehicle (PBS). The graph indicates means and SEMs of 100 organoids from three individual experiments. Round dots represent individual data points. Statistically significant differences were determined by Student's t-test. Scale bars, 10µm.*

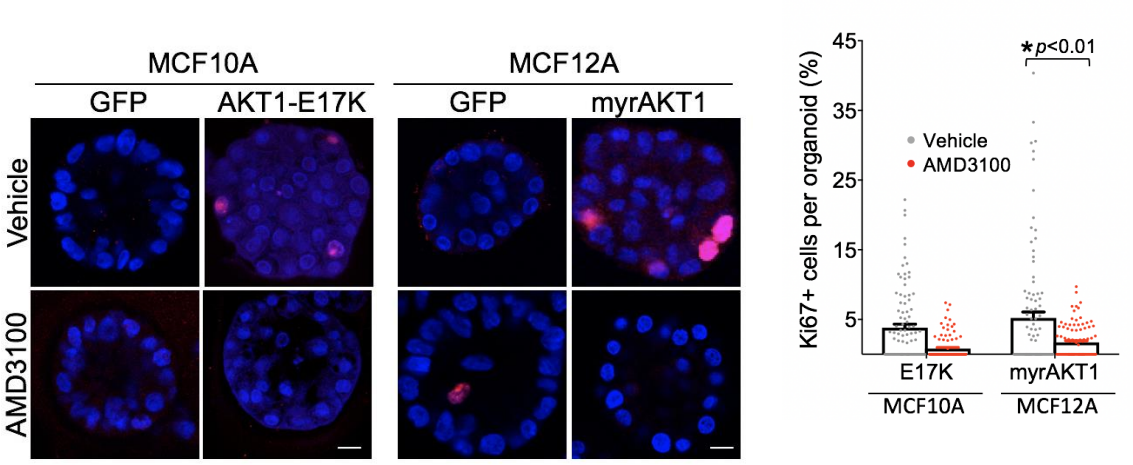

Figure 14: AMD3100 blocks CXCL12-mediated outgrowth in AKT-mutated cells. *Representative images and quantification of cell proliferation in two additional organoid models of quiescent preneoplastic cells with aberrant AKT activation. Growth-arrested organoids derived from MCF10A cells with inducible AKT1-E17K-IRES-GFP or IRES-GFP control and MCF12A cells with inducible myrAKT1-IRES-GFP or IRES-GFP control were treated with CXCL12 in the presence of the CXCR4 inhibitor AMD3100 or vehicle control. The CXCR4 antagonist significantly blocked CXCL12-induced proliferation in both cell models. The graph indicates means and SEMs of 100 organoids from three individual experiments. Round dots represent individual data points. Statistically significant differences were determined by Student's t-test. Scale bars, 10µm.*

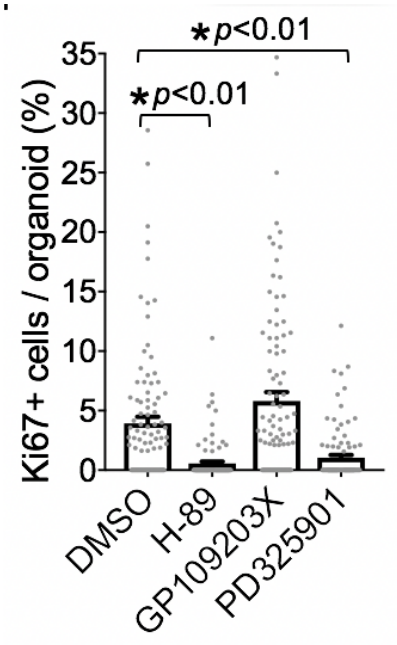

Figure 15: CXCL12 promotes outgrowth through PKA and MEK.

Quantification of AKT-induced preneoplastic cells treated with CXCL12 in the presence of H-89 (PKA inhibitor), GP1092033X (PKC inhibitor), PD325901 (MEK inhibitor), or vehicle (DMSO).

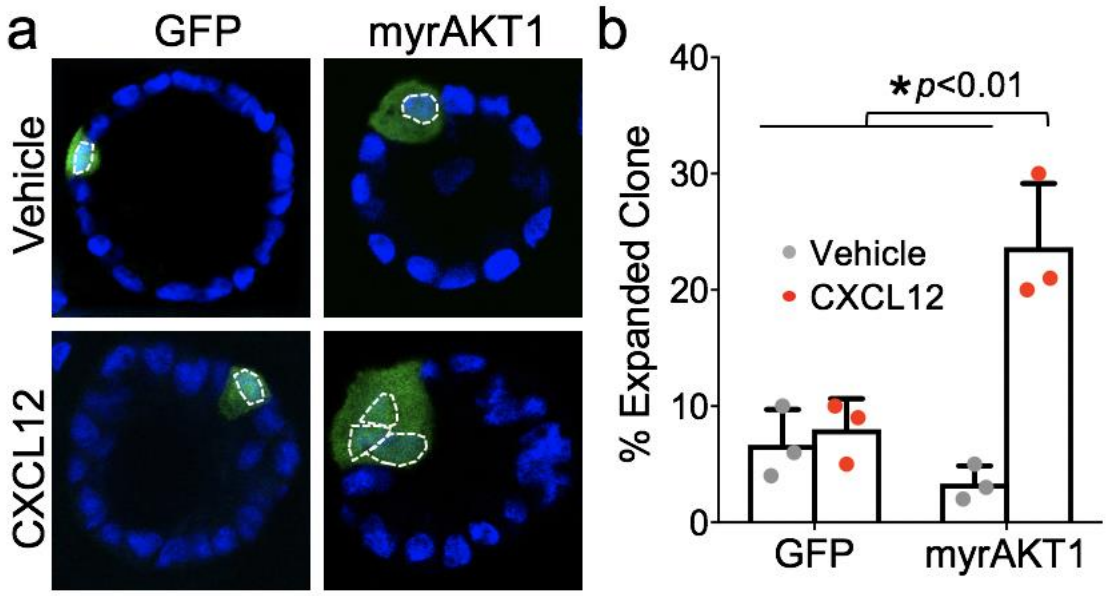

Figure 16: CXCL12 promotes outgrowth of single-infected myrAKT1 cells

***a**) Clonal expansion analysis of transduced single cells in growth-arrested mammary organoids. Nuclei are outlined with dotted white line to aid visualization. **b**) Percent of expanded clones (GFP-positive clusters with more than one nucleus) were determined from 80-120 organoids per sample. Means and standard deviation from three individual experiments were graphed. Round dots represent individual data points. Statistically significant differences were determined by two-tail Student's t-test.*

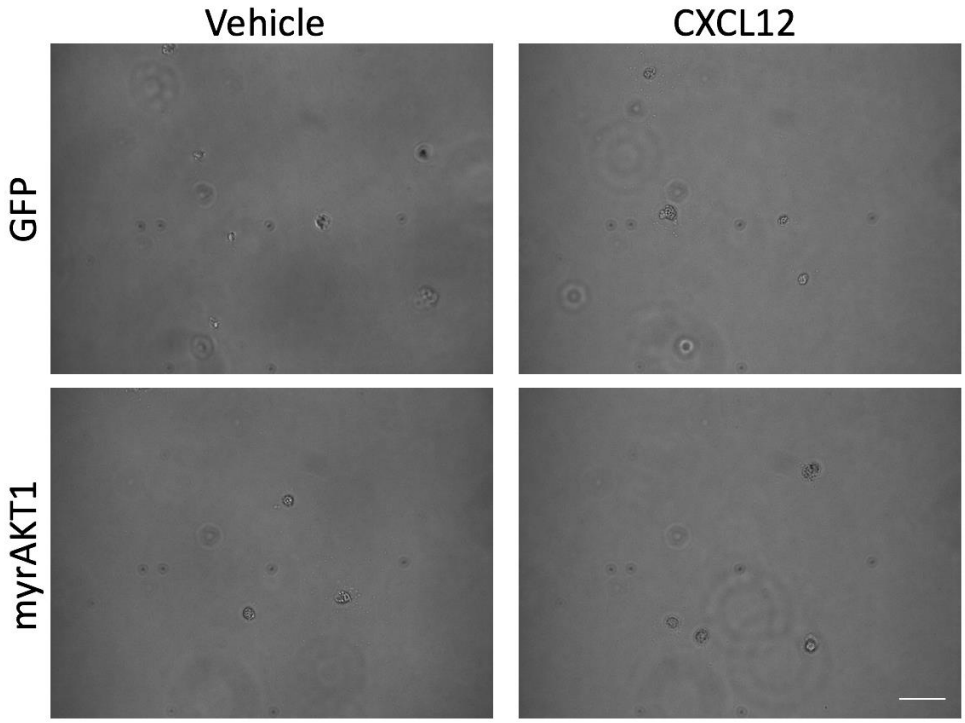

Figure 17: CXCL12-mediated outgrowth does not transform MCF10A cells. Representative images (10x objective) of MCF10A cells with inducible myrAKT1-iGSP or IRES-GFP cultured in soft-agar with media containing 1µg/ml Dox and 50ng/ml CXCL12 or vehicle for 30 days. Cells were plated at 10,000 cells per well of 6-well tissue culture plate. Media were refreshed every 3 days. No colony formation was detected. Scale bar = 50µm

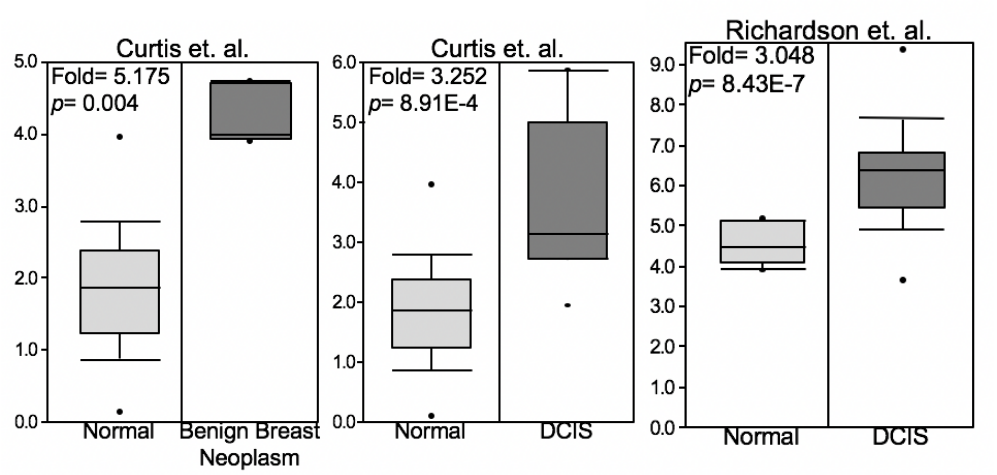

Figure 18: Increased CXCR4 expression correlates with premalignant breast lesions.

Query of CXCR4 mRNA transcript expression in breast samples from Oncomine (ThermoFisher Scientific). Increased CXCR4 expression correlates with premalignant breast lesions.

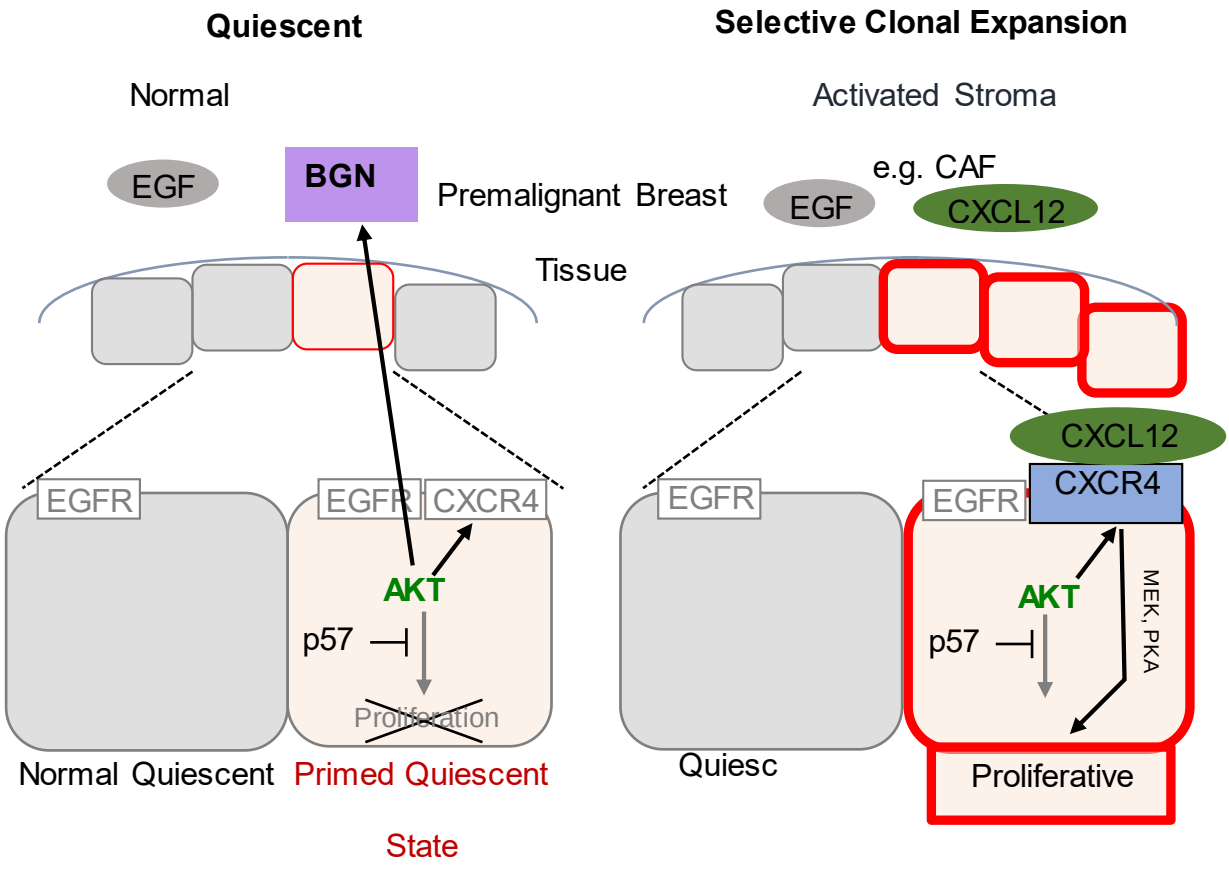

Figure 19: Model of selective clonal expansion of myrAKT1 cells

Model of selective clonal expansion of preneoplastic cells with aberrant AKT activation in permissive stromal environments. Blue, basement membrane. Gray, normal quiescent cells. Pink, primed quiescent state with AKT-induced upregulation of p57Kip2 and CXCR4. Pink with red outline, proliferative preneoplastic cells with aberrant AKT signaling.

Chapter 6: Conclusion

6.1 Introduction

With breast cancer being one of the most commonly diagnosed cancers among women, cancer prevention would have a profound impact on cancer-associated mortality and morbidity. To potentially achieve this, in-depth knowledge of physiological processes underlying the multi-step and linear progression of tumor initiation is required. In principle, tumor prevention would imply the protection against tumor initiation or an efficacious preventative strategy to block the evolution of initiated cells to malignancy. Since there is no way to determine the presence of preneoplastic lesions by reviewing mammograms or other breast imaging tools, understanding how preneoplastic quiescent cells in preneoplastic lesions are able to maintain quiescence while carrying oncogenic changes and understanding what permissive environments allows them to be advantageous in clonal expansion is needed. Creating a more comprehensive picture of the controls and regulations that allow and prevent evolution and clonal expansion of preneoplastic cells into neoplastic lesions and potentially tumors may provide novel markers. These markers could improve detection, diagnosis, and help strengthen current preventative strategies, lowering breast cancer morbidity and mortality.

6.2 Modeling Preneoplastic Quiescence

With cancer models being the primary tool used by cancer researchers in order to test specific hypotheses related to cancer and ultimately gain an overall better understanding of the mechanisms underlying cancer, gaining mechanistic insight into preneoplastic, quiescent cells have been difficult due to a lack of model systems that allow a simplistic systemic manipulation that also recapitulates both the cellular and molecular context of native tissue. Combining genetically altered MCF10A to over-express pro-growth signals with a three-dimensional cell culture system allowed for these cells to model preneoplastic cells in a quiescent state. Furthermore, this model is able to mimic the molecular context unlike traditional cell culture, but also provides a context in which it is feasible to identify genes and dissect mechanisms necessary to produce phenotypic alterations similar to those observed during malignant progression, while also allows for the application of non-genetic factors to investigate cellular mechanisms that govern preneoplastic quiescence, which is not as simple to extrapolate in animal models.

This organotypic system provides a model to study the maintenance of preneoplastic, quiescent cells that contain defined oncogenic alterations. Using cells with aberrant AKT expression as a model for the early genetic changes in preneoplastic cells is representative of all preneoplastic lesions with pro-growth, oncogenic alterations and it allows for insight into the underlying mechanisms of

preneoplastic lesions in general. It also permits convenient genetic manipulation and application of non-genetic factors to investigate cellular mechanisms that govern preneoplastic quiescence. Furthermore, when these quiescent, preneoplastic cells re-enter the cell cycle, the low proliferation rates seen in this model mimic the low proliferation rates seen in preneoplastic lesions. Altogether, these organoid models recapitulate the genetic and proliferative features of quiescent breast preneoplastic cells and, importantly, provide a platform to interrogate the cellular control of quiescent preneoplastic cells at the mechanistic level.

This model system overcomes the major barrier that has impeded investigation of preneoplastic clonal expansion and understanding the underlying mechanisms that regulate and maintain quiescence. Due to its more physiologically relevant environment, a more comprehensive understanding of the regulatory mechanisms and conditions that govern the state of preneoplastic quiescence can be obtained leading to the identification of useful markers of these cells and health conditions. Furthermore, this model system can be used in other contexts beyond what is presented in this thesis research. Other cells lines with other oncogenic changes may be explored more in-depth to form a better understanding of the specific alterations preneoplastic quiescent cells undergo to maintain quiescence and be advantageous in permissive environments. Using this model system to explore other cell lines and oncogenic changes, will help identify more universal preneoplastic markers, as well as, oncogene-specific

markers. Identifying potential markers and developing prognostic markers could be used to evaluate patients' cancer risks potentially leading to a reduction in cancer mortality and develop stronger tumor prevention strategies.

6.3 Maintenance of Quiescence

A critical stage in early breast tumorigenesis is the emergence of preneoplastic cells that carry key tumor drivers but remain in a latent state similar to normal quiescent cells. Such latent preneoplastic cells have been reported in precancerous lesions and tissues surrounding tumors, and are implicated as precursors for primary tumors. Evidence has shown that quiescence is a distinct state, and does not reflect a longer G1 phase. Cells enter quiescence in response to an absence of growth factors or situational cues. It is perplexing that preneoplastic cells can remain in a quiescent state, similar to normal quiescent cells, despite carrying pro-growth genetic alterations. Therefore, understanding and identifying the distinguishing characteristics and signaling mechanisms that underlie the induction and maintenance of preneoplastic quiescence from proliferating preneoplastic cells will provide insights into therapeutic strategies to delay or intervene with the development of tumors.

Taking advantage of the three-dimensional model system, I investigated quiescence maintenance in preneoplastic breast cells with aberrant AKT

signaling. AKT promotes a variety of different functions in the cell including cell survival and cell proliferation. AKT is also a major tumor driver found in early lesions of the breast and may other epithelial tissues. Using aberrant AKT signaling as a model for preneoplastic cells with oncogenic alterations, I discovered cell-intrinsic signaling that played a role in quiescence maintenance.

Overall, quiescent preneoplastic cells with aberrant AKT activation displayed a different genetic expression patterns than both quiescent normal cells and proliferating neoplastic cells with aberrant AKT activation. These results highlight different functional phenotypes. Furthermore, the genetic expression patterns of cell-cycle machinery were altered as well. An increase in p57Kip2 protein and mRNA expression, cyclin-dependent kinase inhibitor, was seen in cells with aberrant AKT signaling. Knocking-down p57Kip2 in these cells was sufficient to drive cell cycle re-entry suggesting that the cell-intrinsic up-regulation of p57Kip2 was to maintain a quiescent state.

Similarly, levels of BGN RNA were significantly higher in quiescent, preneoplastic cells with aberrant AKT activation than both normal quiescent cells and proliferating preneoplastic cells with aberrant AKT activation. This suggests that BGN up-regulation also seems to play a role in quiescent maintenance. Using MCF10A cells with aberrant AKT signaling, I knocked down BGN and saw that quiescence maintenance was disturbed and the cells re-entered the cell cycle. Furthermore, increasing BGN expression in breast tumor cells (T47D) significantly decreased proliferation and colony formation in soft agar assays.

These results suggest that the up-regulation of BGN is another cell-intrinsic signaling mechanism in order to maintain quiescence in preneoplastic cells with aberrant AKT signaling. Although a cell-intrinsic signaling mechanism, it differs from p57Kip2 over-expression due to the fact that BGN is a secreted molecule. Therefore, up-regulation of BGN is being secreted into the microenvironment and modulating the microenvironment to create a niche that prevents clonal expansion.

6.4 Clonal Expansion Advantage

A hallmark of dormancy is the capacity to reverse growth-arrest and enter back into the cell cycle. This ability to exit from a quiescent state is the clinically most relevant phase as the expansion of preneoplastic cells during premalignant development is fundamental to driving clonal evolution and tumorigenesis. Although I gained more understanding into the cell-intrinsic mechanisms that help maintain quiescence, it is unclear what triggers preneoplastic cells with pro-growth, oncogenic alterations to surpass this maintenance and re-enter the cell cycle. Whether a preneoplastic quiescent cell re-enters the cell cycle and evolves into a proliferating cancer cell may not necessarily be only intrinsic, but can depend on environmental cues. Tumorigenic niches have important impacts on this progression. Therefore, a better understanding preneoplastic, quiescent cells

are required for elucidation of biochemical and genetic events involving clonal evolution during tumor development.

Preneoplastic cells and the stromal environment work hand-in-hand to actively drive tumor progression, so I investigated the effects of both normal and cancer-associated fibroblasts on preneoplasic, quiescent cells with aberrant AKT signaling. Previous studies have showed that normal fibroblasts seemed to inhibit proliferation, while CAFs induced cell growth. Results showed an oncogene-mediated switch of mitogen usage promotes selective preneoplastic clonal expansion in permissible microenvironments.

Since EGF is the native growth factor for breast epithelial cells and I had previously shown that there is a change in cell-cycle machinery in quiescent cells with aberrant AKT signaling, I exposed high levels of EGF to these quiescent preneoplastic cells using the three-dimensional model system to see whether higher levels of native growth factor could provoke cell-cycle re-entry. Even with high levels of EGF-exposure, these cells remained in a quiescent state suggesting a homeostatic shift in response to growth stimuli. phenotypes and the altered homeostatic state and response to growth stimuli that quiescent preneoplastic cells displayed. These results are similar to previously reported results suggesting that deregulated EGF signaling is likely to play a role in breast tumor progression. Due to the EGF insensitivity of quiescent cells with aberrant AKT signaling, it is suggested that there is an altered homeostatic state and

response to growth stimuli which may be switched to be sensitive to other growth stimuli.

The critical decision to re-enter the cell cycle is tightly coupled with interactions between preneoplastic cells, host cells, and immune mechanisms. Specific immune effectors and secreted factor (including cytokines and chemokines) have been implicated in the initiation of tumorigenesis and tumor growth. Therefore, using the three-dimensional model system and preneoplastic, quiescent cells with aberrant AKT signaling, I exposed normal and CAF conditioned-media to see whether quiescence maintenance was disturbed since CAFs have been known to be present in preneoplastic and neoplastic microenvironments and play a role in tumor progression. As a result, preneoplastic quiescent cells exited quiescence and showed a significantly higher amount of proliferation. To elucidate what secreted factor was responsible, CAF conditioned-media was looked into further and chemokine CXCL12 showed to mediate cell cycle re-entry of quiescent cells with aberrant AKT signaling. Furthermore, CXCL12-mediated proliferation signaled through the CXCR4 receptor, PKA, and MEK. To determine whether quiescent preneoplastic cells with aberrant AKT signaling were primed for this signaling, I compared CXCR4 expression in preneoplastic quiescent cells with aberrant AKT signaling and normal quiescent cells. Results showed a higher mRNA and protein expression of CXCR4 in quiescent cells with aberrant AKT, suggesting that these preneoplastic cells were primed to have an advantage to clonally expand in this

specific permissive environment. Therefore, although quiescent cells with aberrant AKT activation had cell-intrinsic signals to maintain quiescence, they also expressed a mechanism to make them advantageous in selective clonal expansion driven by CAF-derived chemokine in a premalignant tissue environment.

6.5 Overall Implications

Using the three-dimensional quiescent model together with preneoplastic cells expressing aberrant AKT activation, I was able to gain mechanistic insight into cell-intrinsic homeostatic alterations for quiescence maintenance and also understand mechanisms that gave these cells a clonal expansion advantage in certain environments. Overall, my thesis research has brought to light how complex the system that surrounds preneoplastic cell quiescence is. My results showed that preneoplastic quiescent cells not only alter intrinsically by up- and down-regulating the genetic expression of various proteins, but are able to alter their surrounding environment to help maintain quiescence, as well as, prime themselves to be advantageous in a permissive environment to clonally expand. Although the specifics of my research will not be universal to all preneoplastic lesions, the three overarching themes of intrinsic quiescence maintenance, extrinsic quiescence maintenance, and clonal expansion advantage may be. These three themes should be further explored to gain an understanding of the

specific mechanisms of other underlying oncogenic changes and tissue origins of preneoplastic cells. With this deeper mechanistic insight into quiescent preneoplastic breast cells, novel prognostic markers can be identified which will help improve prevention strategies. In conjunction with underlying oncogenic changes and tissue origins, using novel prognostic markers could help determine whether the lesions in question should be considered more high-risk and low-risk, help improve detection, and make prognosis clearer. From there, the appropriate action can be taken to inhibit or slow down progression, overall improving and strengthening preventative strategies resulting in a decrease in cancer morbidity and mortality.

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